FACTOR V LEIDEN AS RISK FACTOR FOR PREGNANCY COMPLICATIONS

Epidemiological study of Finnish women

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ACADEMIC DISSERTATION

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To my family

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II Hiltunen LM, Laivuori H, Rautanen A, Kaaja R, Kere J, Krusius T, Paunio M, Rasi V. Blood group AB and factor V Leiden as risk factors for pre-eclampsia: A population-based nested case-control study. Thromb Res 2009;124:167-73.

III Hiltunen LM, Laivuori H, Rautanen A, Kaaja R, Kere J, Krusius T, Paunio M, Rasi V. Factor V Leiden as risk factor for unexplained stillbirth – a population-based nested case-control study. Thromb Res 2010;125:505-10.

IV Hiltunen LM, Laivuori H, Rautanen A, Kaaja R, Kere J, Krusius T, Rasi V, Paunio M. Factor V Leiden as risk factor for preterm birth – a population-based nested case-control study. J Thromb Haemost 2011;9:71-8.

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2 ABBREVIATIONS

APC	activated protein C
APC-resistance	resistance to activated protein C
AR	attributable risk
AR%	attributable risk proportion
BMI	body mass index
CI	confidence interval
C4BP	C4b binding protein
DVT	deep venous thrombosis
EPCR	endothelial protein C receptor
F II	prothrombin
F IIa	thrombin
F V	coagulation factor V
F Va	activated factor V
FV Leiden, FVL	factor V Leiden
F VII	coagulation factor VII
F VIIa	activated factor VII
F VIII	coagulation factor VIII
F VIIIa	activated factor VIII
F IX	coagulation factor IX
F IXa	activated factor IX
F X	coagulation factor X
F Xa	activated factor X
F XI	coagulation factor XI
F XIa	activated factor XI
F XIII	coagulation factor XIII
F XIIIa	activated factor XIII
ICD	International Classification of Diseases
IUGR	intrauterine growth restriction
LMWH	low molecular weight heparin
MTHFR	methylenetetrahydrofolate reductase
OR	odds ratio
PAI-1	plasminogen activator inhibitor 1
PAI-2	plasminogen activator inhibitor 2
PAR	population attributable risk
PAR%	population attributable risk proportion
PC	protein C
PROC	protein C
PPROM	preterm premature rupture of membranes
P-value	probability value
PS	protein S
SD	standard deviation
TAFI	thrombin-activatable fibrinolysis inhibitor
TF	tissue factor
TM	thrombomodulin
TFPI	tissue factor pathway inhibitor
VLBW	very low birth weight
VTE	venous thromboembolism
vWF	von Willebrand factor

3 ABSTRACT

Factor V Leiden (FV Leiden) is the most common inherited thrombophilia in Caucasians increasing the risk for venous thrombosis 5-fold. FV Leiden has also been associated with several pregnancy complications. However, the magnitude of risk for pregnancy-associated venous thrombosis needs to be more accurately defined and the impact of FV Leiden on specific pregnancy complications is unclear.

The main aim of the study was to assess FV Leiden as a risk factor for pregnancy complications in which prothrombotic mechanisms may play a part. Specifically, the study aimed to assess the magnitude of the risk, if any, associated with FV Leiden for pregnancy-associated venous thrombosis, pre-eclampsia, unexplained stillbirth, and preterm birth.

The study was conducted as a nested case-control study within a fixed cohort of 100,000 consecutive pregnant women in Finland. The study was approved by the ethics committee of the Finnish Red Cross Blood Service and by the Ministry of Social Affairs and Health. All participants gave written informed consent.

In Finland, practically all pregnant women contact their local Maternity Welfare Clinic during the 8th to 12th week of pregnancy. At the first visit, samples are taken for blood group serology tests, which are performed in the Finnish Red Cross Blood Service at the department of antenatal serology. The department maintains the National Register of Blood Groups and Blood Group Antibodies of Pregnant Women from which data for 100,000 consecutive pregnant women were obtained. Only the first pregnancy of each woman after January 1st, 1997 was included in the cohort. The National Institute for Health and Welfare maintains the National Hospital Discharge Register with diagnoses classified according to the International Classification of Diseases (ICD-10 since 1996). Personal unique identification codes were used to link the two registers to obtain diagnoses for the 100,000 consecutive pregnant women.

The case-candidates and control-candidates who fulfilled the invitation criteria (alive, mother tongue Finnish or Swedish, residence in Finland) were invited by letters and reminders. Participants gave blood samples for DNA tests and filled out questionnaires to supplement clinical data gathered from medical records. The medical records of participants were reviewed in 49 maternity hospitals in Finland. All data were collected on standardized forms blinded to laboratory results. Genomic DNA was isolated from blood samples and genotyping was performed in the Finnish Genome Center. Genotyping of seven polymorphisms, including FV Leiden, was based on restriction enzyme digestions after PCR.

When evaluating pregnancy-associated venous thrombosis, 34 cases and 641 controls were assessed. In all, FV Leiden was associated with an 11-fold risk (OR 11.6, 95% CI 3.6-33.6). When analyzing only cases with the first venous thrombosis, FV Leiden was associated with a 6-fold risk (OR 5.8, 95% CI 1.6-21.8). The risk was modified by blood group, body mass index (BMI), and age. In women with FV Leiden and non-O blood group, the risk was 25-fold compared with women without these characteristics. In women with FV Leiden and BMI over 30 kg/m^2, the risk was 75-fold compared with women without the mutation

and BMI less than 25 kg/m^2. In women with FV Leiden and age over 35 years, the risk was nearly 60-fold compared with women without the mutation and age less than 25 years. In the whole study population, 19% of thromboses were attributable to FV Leiden.

When evaluating pre-eclampsia, 248 cases and 679 controls were assessed. In all, FV Leiden was associated with a trend of increased risk for pre-eclampsia (OR 1.7, 95% CI 0.8-3.9). The point estimates of the risk in subgroups of pre-eclampsia were 1.5-2.5 when all women were analyzed and 2.4-3.4 when primigravid women were considered. However, these associations were not statistically significant.

When evaluating unexplained stillbirth, 44 cases and 776 controls were assessed. In all, FV Leiden was associated with over a 3-fold risk (OR 3.8, 95% CI 1.2-11.6). FV Leiden was especially associated with late unexplained stillbirth with about a 4-fold risk in both all and singleton pregnancies.

When evaluating preterm birth, 324 cases and 752 controls were assessed. In all, FV Leiden was associated with over a 2-fold risk (OR 2.4, 95% CI 1.3-4.6). FV Leiden was especially associated with late preterm birth with about a 3-fold risk, but not with early preterm birth. The association was significant also when primigravid cases and controls were analyzed (OR 3.3) and when cases and controls without stillbirth, pre-eclampsia, intrauterine growth restriction (IUGR), placental abruption, or chorionamnionitis were analyzed (OR 2.6).

This large population-based nested case-control study on ethnically homogeneous women showed FV Leiden to be a clear risk factor for many pregnancy complications. Results were partly confirmatory and partly novel. New information was gained especially on preterm birth and unexplained stillbirth. The results suggest that FV Leiden interacts with common risk factors especially in venous thrombosis. In all, maternal carriage of FV Leiden was associated with an 11-fold risk for pregnancy-associated deep venous thrombosis, a 1.7-fold risk for pre-eclampsia, a 3-fold risk for unexplained stillbirth, and a more than 2-fold risk for preterm birth. The results can be generalized to Finnish women with pregnancies continuing beyond first trimester and may be applied to Caucasian women in populations with similar prevalence of FV Leiden and high standard prenatal care.

4 INTRODUCTION

Thrombophilia means predisposition to thrombosis, i.e., an increased tendency to have blood clots in veins or arteries. Thrombophilia may be inherited or acquired. A point mutation in a coagulation factor V (F V) gene (G1691A), named factor V Leiden (FV Leiden), is the most common known inherited thrombophilia in Caucasians [1]. Due to this mutation, activated F V (F Va) and F V are improperly cleaved and neutralized by activated protein C (APC). This phenomenon is named APC resistance and it leads to enhanced production of thrombin. It is generally accepted that FV Leiden increases the risk for venous thrombosis [2]. However, the extent of the risk in pregnancy-associated venous thrombosis needs to be defined more precisely, considering that pregnancy itself is a hypercoagulable state.

Thrombophilia has been associated, not only with venous thrombosis, but also with many specific pregnancy complications. Normal placental function is vital for fetal wellbeing. It has been hypothesized that thrombophilia may increase the risk for placenta-mediated pregnancy complications (pregnancy loss, pre-eclampsia, IUGR, placental abruption) by two mechanisms: first, by causing placental insufficiency due to placental micro- or macro-vascular thrombosis, and second, by effects on trophoblast cells [3,4]. Inflammatory mechanisms play an important role in both normal and complicated pregnancies [5]. Abnormal immunological balance may lead to pregnancy complications, such as pre-eclampsia and preterm birth [5]. Because of extensive interaction between coagulation and inflammation [6], a third mechanism by which thrombophilia might increase the risk could be through potentiating inflammatory responses. The impact of thrombophilia – including FV Leiden – on specific pregnancy complications is unclear because of conflicting results from mostly small and heterogeneous studies.

Screening for thrombophilia has been under debate since the first findings of association between thrombophilia and pregnancy complications. However, before screening is indicated, a risk factor has to be reliably identified, risk associated with the risk factor should be substantial, and the result of screening should influence treatment [7]. Well-planned epidemiological studies can provide valuable information on the association between a risk factor and disease, in this case, between thrombophilia and pregnancy complications. Nested case-control study design, a variation of cohort study, has the advantages of a cohort study and is more feasible as only cases and a sample of controls in a fixed cohort are studied in detail [8].

In Finland, conditions for population-based studies are good due to high-quality administrative national registers (e.g., the Hospital Discharge Register) and a possibility to link data from different registers by using unique identification codes. Finland has a comprehensive free prenatal care system ensuring that pregnancy complications are diagnosed early and treated properly. Practically all pregnant women contact the Maternity Welfare Clinic during the first trimester of pregnancy and are thereby registered in the National Register of Blood Groups and Blood Group Antibodies of Pregnant Women kept by the Finnish Red Cross Blood Service. The Finnish population is ethnically homogeneous which enables the unconfounded assessment of genetic risk factors that are of Caucasian origin.

The importance of FV Leiden as a risk factor for pregnancy complications clearly needs further investigation. In this population-based nested case-control study, FV Leiden was assessed as a risk factor for pregnancy-related venous thrombosis, pre-eclampsia, stillbirth, and preterm birth in a large cohort of 100,000 consecutive pregnant Finnish women. Cases and controls were identified by linking national registers. Information gathered from the questionnaires and medical records of participants made it possible to ensure the accuracy of register-based diagnoses and to analyze clinical subgroups.

5 REVIEW OF THE LITERATURE

5.1 Hemostasis

The main factors maintaining the balance between bleeding and thrombosis are the vessel wall, platelets, coagulation system, and fibrinolytic system.

At the site of a vessel wall injury, platelets serve as the first hemostatic plug by adhering to exposed collagen directly and through von Willebrand factor. Aggregated and activated platelets support local coagulation by providing a negatively charged phospholipid surface for the coagulation cascade, which eventually forms a stable fibrin clot. Coagulation is regulated by natural anticoagulant mechanisms to limit the process at the site of injury. Finally, the clot is dissolved by the fibrinolytic system. [9]

5.1.1 Coagulation cascade

Figure 1 presents a sketch of the coagulation cascade. The procoagulant coagulation cascade is composed of serine protease enzymes and their cofactors. The end point of this cascade is the formation of active thrombin. The coagulation cascade occurs on a phospholipid surface, mainly on the activated platelets or the injured endothelium, in the presence of Ca++. The coagulation process begins when tissue factor (TF) is exposed to blood and binds with F VIIa, which pre-exists in trace amounts in the blood. F VIIa needs to be bound to TF to gain proteolytic activity. TF - F VIIa complex activates F IX and more efficiently F X. [10] The first small amounts of F Xa activate F V, and together they form a prothrombinase complex to activate prothrombin to thrombin [11]. After this initiation phase, the newly formed thrombin activates F V, F VIII, and F XI, thereby accelerating its own activation and leading to a very efficient propagation phase of coagulation. F IXa, with its now activated cofactor F VIIIa (tenase complex), activates efficiently F X, and then F Xa, with its cofactor F Va (prothrombinase complex), activates prothrombin to thrombin. F XIa serves as another activator for F IX to ensure the efficiency of the thrombin formation process. [10] Thrombin converts the soluble fibrinogen into insoluble fibrin, which forms a network in and around the platelet plug. Thrombin also activates F XIII, which cross-links fibrin molecules to form a stable clot. [9] In addition, thrombin further activates platelets [10], ensuring excellent conditions for coagulation to proceed on the phospholipid surface.

As a link between coagulation and inflammation, thrombin can activate endothelial cells, mononuclear cells, platelets, fibroblasts, and smooth muscle cells through PAR-1, PAR-3, and PAR-4 (protease activated reseptors) on their surface, leading to the production of several cytokines and growth factors [6].

Anticoagulant mechanisms regulate the coagulation cascade rigorously to limit thrombosis at the site of vessel wall trauma. Limiting factors include several phenomena: adhered, activated platelets remain at the site of injury, serine proteases involved in the process need to be proteolytically activated, and physiologic anticoagulants – tissue factor pathway inhibitor (TFPI), antithrombin, and the protein C system – control critical points of the coagulation cascade [10].

Platelet factor 4 released from platelets increases protein C activation rate and this may also limit thrombus formation outside the site of injury [12].

TFPI neutralizes stoichiometrically the TF - F VII complex [10]. Antithrombin can neutralize all the procoagulant serine proteases by binding to them [10], the primary targets being thrombin, F Xa, and F IXa [13]. The protein C system regulates the coagulation process dynamically by responding to the presence of thrombin. This anticoagulant system is described in detail in the next section.

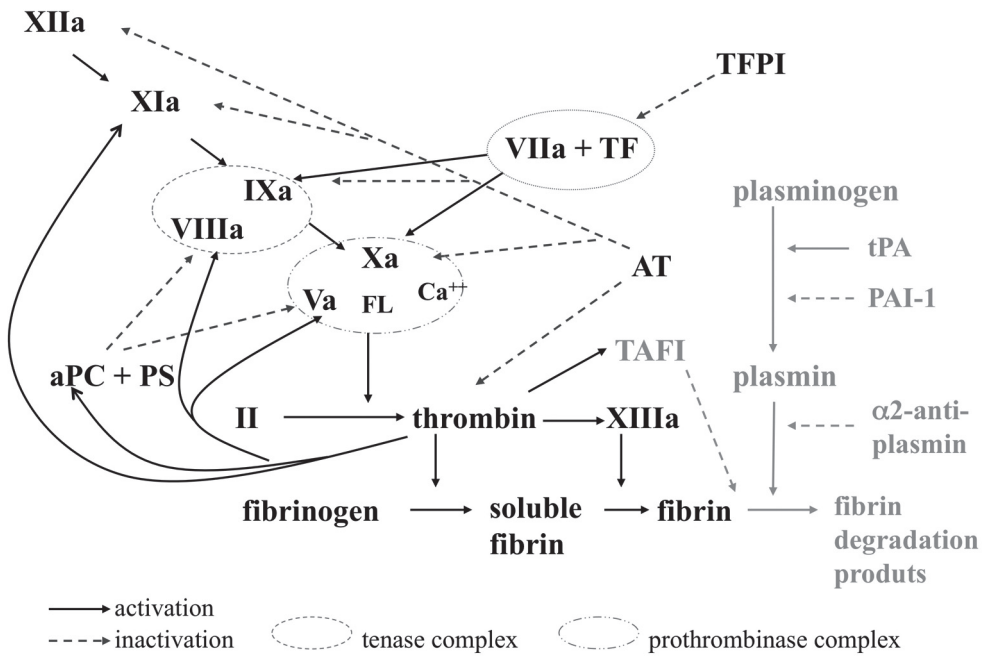


Figure 1. *Coagulation cascade.*

5.1.2 Protein C anticoagulant pathway

After thrombin is formed, it down-regulates its own formation through the thrombin-thrombomodulin-protein C system [10]. When thrombin binds to thrombomodulin present on the surface of the intact endothelium, it loses its procoagulant activity. Thrombomodulin-bound thrombin is not only efficiently inactivated by antithrombin and other inhibitors, but it also activates protein C to activated protein C (APC) [12]. Endothelial cell protein C receptor (EPCR), also present on the endothelium, presents protein C to the thrombin-thrombomodulin complex enhancing protein C activation [12].

APC, with its cofactor protein S, inactivates F Va and F V IIIa by cleaving certain peptide bonds in them. F Va is cleaved at least at the sites R306, R506, and R679 and F VIIIa at the sites R336 and R562 [14]. This inactivation of central factors in the propagation phase of the coagulation cascade efficiently reduces the formation of thrombin and eventually also the formation of APC. APC is slowly inactivated by protein C inhibitor and alfa-1 antitrypsin [14].

The thrombin-thrombomodulin complex efficiently activates also thrombin activatable fibrinolysis inhibitor (TAFI), which renders fibrin clot more resistant to lysis [13]. The protein C pathway is also involved in limiting inflammatory responses [6,12].

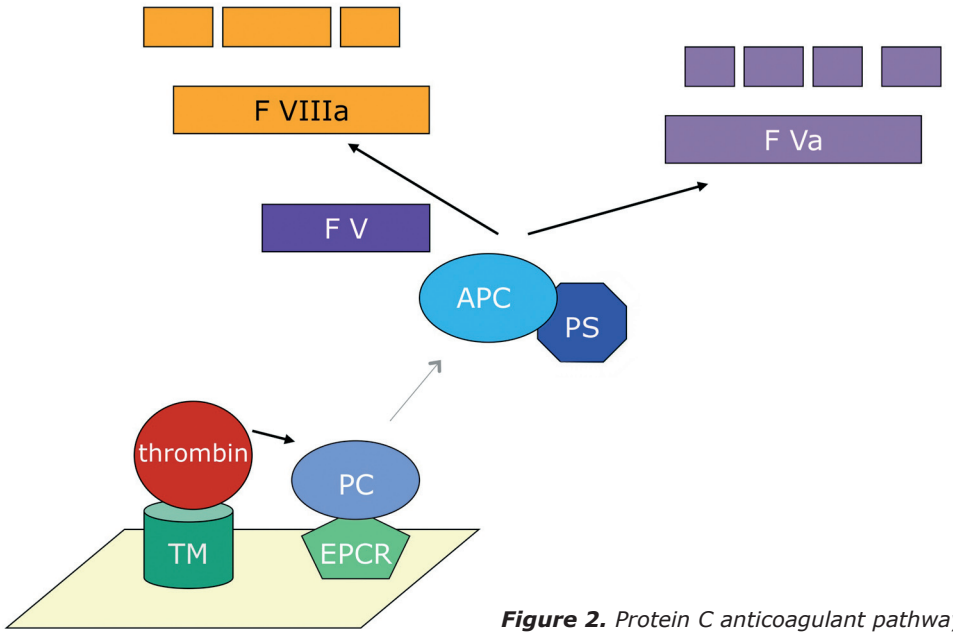


Figure 2. *Protein C anticoagulant pathway.*

5.1.3 Factor V

Factor V (F V), which was discovered by Paul Owren in 1943 [15], has proved to be an important regulator of the hemostatic balance with both procoagulant and anticoagulant properties [14].

The gene of F V is on the chromosome 1 (1q23), and this single-chained glycoprotein of 2,196 amino acids is synthesized in the liver. Of the total F V, 20% is stored in platelet α-granules, the rest circulates in plasma [11]. The F V in platelets is of plasma origin, but it is already modified in platelets by partial proteolysis, giving it considerable F Xa-cofactor activity [11]. This seems to be an efficient way to ensure that this important factor is immediately present at the site of vessel wall injury and ready to function.

F V is activated by F Xa or thrombin to F Va by the cleavage of three peptide bonds (Arg709, Arg1018, Arg1545) [11]. The inactivation of F Va is mediated through APC, which cleaves the F Va at the sites Arg506, Arg306, and Arg679,

usually in this order [14]. The Arg506 is the preferred site for proteolysis, but protected by F Xa in prothrombinase complex when coagulation is in process. However, protein S accelerates the slower proteolysis at the site Arg306 [16] and helps APC to reach the Arg506 site [13]. After cleavage at the site Arg506, F Va still has partial procoagulant activity, which is abolished when the Arg306 and Arg679 peptide bonds are cleaved [17].

F V has procoagulant as well as anticoagulant properties. In its activated form, F Va serves as an essential cofactor for F Xa (the prothrombinase complex) in the formation of thrombin [11]. On the other hand, the intact F V acts as a cofactor in the protein C system by stimulating the cofactor activity of protein S in the inactivation of F VIIIa by APC [18]. This anticoagulant activity appears after the cleavage of a peptide bond at the Arg506 by APC [14]. Mutations in the F V gene may lead to hemorrhagic and thrombotic tendencies.

5.2 Pregnancy and hemostasis

Many adjustments and adaptations happen in a woman during pregnancy. In the blood, the most important alterations during normal pregnancy are increased plasma volume, physiologic decrease of hemoglobin, occasional mild thrombocytopenia, neutrophilia, increases in many procoagulant factors, and attenuated fibrinolysis [19].

Increases in many coagulation factor levels, decrease of anticoagulant activity, and diminished fibrinolysis lead to a hypercoagulable state protecting from excessive bleeding during delivery. The most prominent changes are a decrease in protein S activity (due to the increase of C4BP); acquired protein C resistance; increased levels of von Willebrand factor, F VIII, and F VII; increased fibrinogen; and increased activity of fibrinolytic inhibitors (TAFI, PAI-1, PAI-2). Usually the levels of F II, F V, F IX, and F X increase slightly and the level of F XI decreases slightly. [19-22] F XIII level increases early in the pregnancy but decreases thereafter [20]. Coagulation parameters usually reach their baseline levels by eight weeks postpartum [19].

5.3 Factor V Leiden

5.3.1 History

In 1993, Dahlbäck *et al.* in Malmö, Sweden described a new phenomenon, i.e., poor anticoagulant response to activated protein C, in a family with a history of venous thromboses. The phenomenon was thought to be due to the deficiency of a new protein C cofactor and the laboratory phenomenon was named APC resistance [23]. In May 1993, a commercial APC resistance test became available.

APC resistance was quickly demonstrated to be a common risk factor for venous thrombosis. In the Leiden Thrombophilia Study (LETS), APC resistance was present in 21% of venous thrombosis patients and in 3 % of controls [24]. In a Swedish material, about 40% of 104 consecutive venous thrombosis patients had APC resistance compared with 7 % of controls [25]. APC resistance was

also shown to be present in over 50% of previously unexplained thrombophilic patients and concomitantly in a few with a previous diagnosis of protein C or protein S deficiency [26].

In early 1994, Dahlbäck *et al.* reported the cause of APC resistance to be a property of factor V [27]. At the same time, Bertina *et al.* in Leiden, The Netherlands also concluded that F V was involved. In June 1994, they published a paper showing that a single G to A substitution at the nucleotide position 1691 in the factor V gene was associated with APC resistance [1]. The point mutation causes the replacement of amino acid Arg to Gln at the site 506 in factor V resulting in the inadequate inactivation of mutated F Va. The mutation was named as factor V Leiden (FV Leiden) [1]. As the site Arg506 is a cleaving site for APC, it was now easy to understand why the disappearance of this site can cause resistance to APC. Dahlbäck's initial idea of the lack of a new protein C cofactor behind APC resistance has also proved to be partially true as the FV Leiden mutation abolishes the cofactor activity of F V in the inactivation of F VIIIa [14].

Dahlbäck and Zöller *et al.* found FV Leiden mutation in almost all of their fifty Swedish APC resistant families proving this mutation to be the most prevalent cause for APC resistance [28]. The first large study, LETS, already proved FV Leiden to be a frequent risk factor for venous thrombosis [29].

5.3.2 Prothrombotic mutation: gain of function – loss of function

FV Leiden is a prothrombotic mutation. It is at the same time a gain-of-function mutation and a loss-of-function mutation. First, due to disappearance of the cleavage site at the Arg506, APC is unable to inactivate F Va optimally leading to increased thrombin formation [17]. Second, due to the disappearance of that cleavage site, APC is unable to cleave intact F V so that F V could function as cofactor for PC-PS complex in the inactivation of F VIIIa. This loss of anticoagulant function leads, again, to increased thrombin formation [14].

However, the risk for venous thrombosis caused by FV Leiden is relatively low. This may be explained by the fact that although cleavage at the site Arg506 accelerates inactivation of F Va remarkably by exposing the cleavage sites Arg306 and Arg679 to APC, cleavage at the site Arg506 is not absolutely necessary for the inactivation of F Va [17]. In addition, in the prothrombinase complex, the capability of APC to inactivate F Va is similar for the wild type F Va and F Va Leiden, because the Arg506 cleavage site of the wild type F Va is protected by F Xa, and in F Va Leiden this cleavage site does not exist. [16,17]. Mechanisms that reduce the effect of this potentially injurious mutation include the acceleration of the cleavage of F Va by protein S at the site Arg306 [16].

5.3.3 Epidemiology and evolutionary advantage

According to haplotype analyses, FV Leiden is a founder mutation, which occurred about 21,000 years ago [30-32]. The mutation is present at a variable frequency (mean 5%) in Caucasians, but absent or nearly absent in other races [33-35]. This indicates that FV Leiden most likely occurred in a Caucasoid subpopulation after the separation of non-Africans from Africans, and Caucasoid populations from Mongoloid populations [31,33,34].

The high prevalence of FV Leiden in Caucasians suggests an association with evolutionary advantage and many findings support a favourable selection pressure [34]. Data exist indicating that FV Leiden might protect against peripartal bleeding [36-38] and heavy menstrual blood loss [39]. This could have provided considerable advantage by reducing iron depletion and by protecting against life-threatening post-partum hemorrhage. However, conflicting observations about pregnancy-related blood loss exist [40]. Similarly to protecting against excessive bleeding in association with surgery [41], FV Leiden may have protected against excessive bleeding in association with trauma in the past. Some evidence, although partly conflicting, exists that simultaneous carriage of FV Leiden might attenuate bleeding symptoms also in hemophiliacs [42].

Other possible selective advantages include a more favourable embryo implantation in carriers of FV Leiden [43,44], and an increased fecundity (shorter time to pregnancy) in the male carriers of FV Leiden [45]. This is supported by an observation of a slightly increased sperm count in the male carriers of FV Leiden [46].

5.4 F II G20210A

Poort *et al.* reported in 1996 a point mutation in the coagulation factor II (prothrombin) gene [47]. The mutation causes a G to A substitution at the nucleotide position of 20210 in the 3'-untranslated region of the gene. The point mutation is associated with elevated prothrombin levels and is therefore a gain of function mutation. FII G20210A allele is associated with about a 2-fold increased risk for venous thrombosis [47]. The mutation is of single origin and is mainly found in the Caucasian population [32].

5.5 Assessment of risk associated with a genetic risk factor

Several different study designs can be used to assess the association between a specific genetic risk factor and a disease entity. Each design has its advantages and limitations.

In *case-control studies*, cases are selected on the basis of developing a specific disease (outcome). The disease entity should be as homogeneous as possible to minimize the risk of any true association remaining unobserved. Controls should be from the same population as cases, i.e., if a person without the disease had developed the disease, she/he would have been selected as a case. The case-control design is particularly suitable for rare diseases and it allows many risk factors to be evaluated simultaneously. Being less expensive and time-consuming than cohort studies, case-control studies are often more feasible. However, they are susceptible to selection bias (inclusion of cases or controls is somehow dependent of the studied risk factor) and information bias (knowledge of disease status, recall bias, reporting bias, research bias, misclassification). Therefore, studies should be carefully planned to avoid these biases. Well-planned and conducted case-control studies can provide valuable information on the association between a risk factor and disease and they can be reliably used to test epidemiologic hypothesis. [48]

In case of genetic risk factors, case-control studies are efficient and reliable in estimating risks if their sizes are in accordance with the prevalence of the studied mutation, i.e., if they have enough statistical power [49]. However, false positive and false negative associations are possible if the studied population includes genetically heterogeneous subgroups [50]. Genetic association studies cannot prove causality as the studied genetic marker may only be linked to the causative genetic factor.

In *cohort studies*, individuals are selected on the basis of having or not having an exposure or risk factor. Exposed and unexposed individuals are then followed to assess the risk for an endpoint or disease. The exposed and unexposed should be as similar as possible except for the studied risk factor. The cohort design is particularly suitable when the risk factor is rare. Also, it allows assessment of many endpoints for a single exposure and direct calculation of endpoint incidence rates in the exposed and unexposed. [8]

The best way to establish whether and how much a single mutation alters the risk for a specific disease is to study the absolute risk of the disease in carriers and non-carriers of the mutation in a fixed population-based cohort over a defined time. *Prospective cohort studies* may have the lowest risk for selection bias as the cohort has been identified before the development of the disease. However, these studies are seldom feasible as large cohort studies needed for rare diseases can be extremely expensive and time-consuming [8].

A more feasible variation of a cohort study is a *nested case-control study* in which only cases and a sample of controls in a fixed cohort are assessed in detail [8]. In genetic association studies with this design, only cases and controls are genotyped for the studied mutation. In this setting, it is possible to study relative risks and their ratios and even population parameters that are readily generalizable to the known reference population if the sampling is unbiased.

Sometimes the term retrospective study is used as a synonym for a case-control study, because in this design researchers have first an outcome for which they aim to ascertain a cause. Analogously the term prospective study is sometimes used as a synonym for a cohort study, because in this design researches have first a suspected risk factor and they follow up a cohort for an outcome. However, the terms retrospective and prospective are often used to define whether the outcome has occurred before or after the study started. Therefore, case-control and cohort studies can be either retrospective or prospective, although this distinction is usually used only for cohort studies. [48]

In all epidemiological studies, it is vital that information has been gathered identically from all study subjects. Information about exposure and outcome should be accurate and complete [48]. When the information is gathered retrospectively, adequate records should be available, and sometimes several sources may have to be used [8]. Whether the risk has been assessed in family studies, hospital-based studies, registry-based studies, or population-based studies, the populations the results can be generalized to must be carefully considered.

In case of thrombophilia, cohorts of carriers (exposed) and non-carriers (unexposed) of a mutation are most readily available from thrombophilic families. However, population-based studies give more generalizable results. In hospital-based studies, cases often represent the most severe cases of the specific disease, which may distort the results leading to an overestimation of

the risk associated with the mutation. Register-based studies are also used in genetic association studies. They are feasible but only as accurate and reliable as the information in the registers. Therefore, the validity of diagnoses in the register is of great importance [51]. Registers can be used to identify cohorts or cases and controls, which then are recruited for the study to give samples for DNA. Register-based studies become laborious, but also more accurate, when diagnoses and clinical data are checked from the medical records.

As in all research, possible publication-bias should be kept in mind when reviewing the literature about genetic risk factors. Publication bias exists when researchers, reviewers, or editors submit or accept papers for publication depending on the direction or strength of the results [52].

5.6 Venous thromboembolism

Venous thrombosis can be seen as a classic example of complex common disease which is caused by interaction of acquired and inherited risk factors [49]. Thrombosis occurs when many risk factors are simultaneously present. Each risk factor increases the thrombotic potential and eventually a trigger point for thrombosis is exceeded. The risk of thrombosis increases with age. Therefore, in young adults more risk factors are needed for thrombosis to occur than in old age. Among women of fertile-age the incidence of thrombosis is about 1:10,000 women years. [53]

According to Virchow's triad from the 1856, the emergence of thrombosis is due to changes in the vessel wall, in blood, and in the velocity of blood flow [14]. Venous and arterial thromboses only partly share the same risk factors and both also have their own risk factors [54].

Thrombophilia can be inherited or acquired. Defects of natural inhibitors of coagulation or gain of function of coagulation factors can disturb the strictly regulated balance to favour thrombus extension. Antithrombin deficiency, protein C deficiency, and protein S deficiency are well known, although rare, inherited risk factors for venous thrombosis. They are strong risk factors for venous thrombosis, the estimated increase of risk being about 10-fold [2]. Their impact on arterial thrombosis, however, is marginal [54].

Gain-of-function mutations, FV Leiden and prothrombin G20210A (FII G20210A), are moderate risk factors for venous thrombosis, increasing the risk 5-fold and 2- to 3-fold, respectively [2]. They do not have a major impact on arterial thrombosis, although in special subgroups of young patients they may be involved to some extent [55].

For acquired thrombophilia, antiphopholipid antibodies are of great importance. Antiphospholipid antibodies are risk factors for venous and arterial thrombosis as well as for pregnancy complications [54].

Non-O blood group is associated with a 2- to 4-fold increased risk for venous thrombosis compared with blood group O [2,56]. This is probably due to the higher levels of von Willebrand factor (vWF) and F VIII in individuals with these blood groups. The lower level of vWF in individuals with blood group O may be due to more efficient clearance of vWF, which may be determined by ABH antigens on vWF [57].

The increasing thrombosis risk associated with increasing age may be due to the progressive increase of many coagulation factors, impaired function of fibrinolytic system, and age-related structural and functional changes in vessel walls [54]. Other recognized acquired risk factors for venous thrombosis include obesity, previous venous thrombosis, surgery, trauma, immobilization, cancer, oral contraceptives, hormone replacement therapy, and pregnancy [54].

5.6.1 Venous thromboembolism in pregnancy

Pregnancy-associated venous thromboembolism is a rare cause of maternal morbidity occurring in less than 1 in 1,000 pregnancies in western countries [58-62]. In these countries, it is, however, a major cause of maternal mortality [58,63-65]. In Finland also, thromboembolism is the main cause of maternal deaths [66,67].

Pregnancy increases the risk for venous thrombosis 4- to 10-fold. Besides being a hypercoagulable state, pregnancy causes venous stasis in lower extremities due to the enlarged uterus, and during labour the endothelium of pelvic vessels may be damaged. Thromboses in the veins of the left lower extremity are overrepresented compared with thromboses occurring in non-pregnant state. This may be due to the pronounced compression of the left iliac vein by the right iliac artery. Most pregnancy-related venous thromboses occur during pregnancy, but the risk for venous thrombosis is higher in postpartum period. [58,63,68]

5.6.2 FV Leiden and venous thromboembolism in pregnancy

Studies that assess the risk associated with FV Leiden for pregnancy-associated venous thromboembolism (VTE) vary in many respects. Study designs, selection of cases and controls, reporting of ethnicity, definition of puerperium (from 3 weeks to 3 months postpartum), inclusion of recurrent VTE events, and inclusion of homozygotes in analyses differ. Case-control and cohort studies are summarized in tables 1 and 2.

In case-control studies (table 1), the odds ratio of pregnancy-associated venous thromboembolism for FV Leiden varies from 2.8 to 18.3.

In a meta-analysis published in 2006 by Biron-Andreani *et al.* [69], a pooled OR of six case-control studies was 8.6 (95% CI 5.9-12.6), although these studies were found to be heterogeneous. Studies included in this meta-analysis are marked with # in the table 1.

In cohort studies (table 2), the odds ratio of pregnancy-associated venous thromboembolism for mainly heterozygous FV Leiden varies from 3.7 to 8.3. [37,80-84]. For homozygous FV Leiden the OR has been 41.3 [85].

In the meta-analysis by Biron-Andreani *et al.* [69], a pooled OR of cohort studies was 4.5 (95% CI 1.8-10.9). This meta-analysis included cohorts from thrombophilic families [80,81,83] as well as prospective cohorts of pregnant women [37,84]. Studies included in this meta-analysis are marked with # in the table 2.

Prospective population-based cohort studies would give the best estimation of the risk associated with FV Leiden in general population. However, the three prospective studies available [37,82,84] consist of less than 4,700 mainly White women, of whom only 383 are carriers of FVL. Numbers are too small to give a definite estimate of risk given that pregnancy-associated venous thrombosis is so rare, usually less than 1 per 1,000 pregnancies.

In a systematic review and meta-analysis of Robertson *et al.* [79], heterozygous and homozygous carriers of FV Leiden were analyzed separately for the risk of pregnancy-associated venous thrombosis. The pooled OR was 8.3 (95% CI 5.4-12.7) for heterozygotes, and 34.4 (95% CI 9.9-120) for homozygotes. There were no signs of heterogeneity although the eight studies included case-control and cohort studies, as well as family studies. Studies included in this meta-analysis are marked with ¤ in the tables 1 and 2.

Taken together, FV Leiden has been consistently associated with an increased risk for pregnancy-associated venous thrombosis. However, population-based studies are still needed to assess the risk in carriers of FV Leiden from the general population.

Table 1. *FV Leiden and risk for venous thrombosis associated with pregnancy. Case-control studies.*

Study	Country	Self-reported study design	Study population	Cases
McColl *et al.* 1997 [59]	UK; Two maternity units	Retrospective	Ethnicity not reported	50
Grandone *et al.* 1998 [70] #¤	Italy; Two thrombosis centers	Case-control	White women	42
McColl *et al.* 2000 [71]	UK; Two maternity units	Retrospective	Ethnicity not reported	75
Gerhardt *et al.* 2000 [72] #¤	Germany; University medical center	Case-control	Ethnicity not reported	119
Dilley *et al.* 2000 [73] #¤	US; Four hospitals	Case-control, retrospective	76 White (41 Black)	27
Martinelli *et al.* 2002 [74] #¤	Italy; Two thrombosis centers	Case-control	Caucasians; women with antiphospholipid antibodies excluded	119
Gerhardt *et al.* 2003 [75] #	Germany; University medical center	Retrospective case-Control	Ethnicity not reported	190
Meglic *et al.* 2003 [76] #	Slovenia; One center	Retrospective case-control	Ethnicity not reported	30
Pomp *et al.* 2008 [77]	The Netherlands; Six anticoagulation clinics	Population-based case-control	Ethnicity not reported; age<50 years, no P-pills or HRT	285; consecutive patients with first VTE
Jacobsen *et al.* 2010 [78]	Norway	Population-based case-control	Women with 23 completed weeks of pregnancy; registry-based identification of participants; Norwegians	313 (from 18 hospitals)

* Calculated from data given in article (StatsDirect).

Study included in meta-analysis by Biron-Andreani *et al.* [69].

¤ Study included in meta-analysis by Robertson *et al.* [79].

Controls	Venous thrombosis	Prevalence of FVL	OR (95% CI)
Population prevalence as control	Objectively diagnosed DVT during pregnancy or puerperium	Cases: 4/50, 8.0% Population: 3.0%	2.8*
213; parous, age-matched	Objectively diagnosed DVT during pregnancy or puerperium	Cases: 10/42, 23.8% Controls: 4/213, 1.9% (all heterozygous)	16.3 (4.8-54.9)
221; General population	Consecutive; objectively diagnosed DVT during pregnancy or puerperium	Cases: 7/75, 9.3% Controls: 5/221, 2.3% (homozygotes included)	4.5 (2.1-14.5)
233; blood donors, age-matched, 157 parous	History of objectively diagnosed DVT during pregnancy or puerperium; (23 had recurrent DVT)	Cases: 52/119, 43.7% Controls: 18/233, 7.7% First DVT: Cases: 34/79, 43.0% (homozygotes included)	9.3 (5.1-16.9) First DVT: 9.0 (4.7-17.4)
49; matched for hospital (and race)	Objectively diagnosed VTE during pregnancy or puerperium (one had history of VTE)	Cases: 8/27, 29.6% Controls: 1/49, 2.0% (homozygotes included)	18.3 (2.7-432)
232; parous	First DVT during pregnancy or puerperium; objectively diagnosed	Cases: 22/119, 18.5% Controls: 6/232, 2.6% (figures for heterozygotes)	10.6 (5.6-20.4)
190; parous blood donors, age-matched, same region	First DVT during pregnancy or puerperium; objectively diagnosed	Cases: 50/166, 30.1% Controls: 11/187, 5.9% (figures for heterozygotes)	6.9 (3.5-13.8)
56; age-matched, delivery in same hospital during same fortnight	Objectively diagnosed VTE during pregnancy or puerperium	Cases: 8/30, 26.7% Controls: 3/56, 5.7% (all heterozygous)	5.5 (1.2-24.8)
857; partners of cases and a random sample	Objectively diagnosed VTE in 90% of patients	Pregnant cases: 19/100, 19.0% Pregnant controls: 3/59, 5.1%	4.4 (1.2-24)* (FVL and pregnancy/ puerperium vs. non-pregnant non-carriers: 52 (12-220))
353 (from one university central hospital)	First DVT during pregnancy or puerperium	Cases: 74/313, 23.6% Controls: 23/353, 6.5% (figures for heterozygotes)	5.0 (3.1-8.3)

Table 2. *FV Leiden and risk for venous thrombosis associated with pregnancy. Cohort studies.*

Study	Country	Self-reported study design	Study population	Carriers of FVL
Simioni *et al.* 1999 [80] #	Italy	Retrospective family cohort study	Family members of probands with documented DVT and FVL; ethnicity not reported	224
Lindqvist *et al.* 1999 [37] #	Sweden	Prospective (population-based cohort) study	2,480 pregnant women; Swedish (31 % non-Swedish)	270 APC resistant pregnant women (FVL confirmed)
Lensen *et al.* 2000 [81] #	The Netherlands	Retrospective follow-up (family) study	Family members of probands with documented DVT, positive family history for DVT, and FVL; ethnicity not reported	47 women with 100 pregnancies
Murphy *et al.* 2000 [82] ¤	Ireland (two clinics)	Prospective cohort study // Retrospective observational study	588 unselected primigravid women without history of thrombosis or hypertension // Women with history of DVT during pregnancy; ethnicity not reported	16
Pabinger *et al.* 2000 [86] ¤	Austria, Hungary, Germany (five "thrombophilia centers")	Multicenter retrospective cross-sectional study	Ethnicity not reported	64 homozygotes; 212 pregnancies
Martinelli *et al.* 2001 [85] ¤	Italy	Multicenter retrospective family study	Relatives of probands, ethnicity not reported	9 homozygous women; 19 pregnancies
Tormere *et al*. 2001 [83] #¤	Italy, one center	Retrospective family cohort study	Parous family members of probands with documented DVT and FVL; ethnicity not reported	94 heterozygotes; 6 homozygotes
Dizon-Thompson *et al.* 2005 [84] #	US; 13 centers	Prospective observational multicenter study	4,885 (1,602 White) pregnant women (exclusion: multiple pregnancy, history of VTE, anticoagulant therapy, known FVL status or antiphospholipid syndrome)	134 (White 97)

* Calculated from data given in article (StatsDirect).

\# Study included in meta-analysis by Biron-Andreani *et al.* [69].

¤ Study included in meta-analysis by Robertson *et al.* [79].

Non-carriers of FVL	Venous thrombosis	Results	OR (95% CI)
154	Documented DVT during pregnancy or puerperium	FVL carriers: 3 DVT in association with 157 pregnancies FVL non-carriers: 0 DVT in 93 pregnancies (homozygotes included)	4.2 (0.5-148)
2,210 non-APC resistant pregnant women	DVT during pregnancy or puerperium (history of VTE in 9)	3/270 FVL carriers had VTE 3/2,210 FVL non-carriers had VTE (homozygotes included)	8.3 (1.7-41.2)
44 women with 50 pregnancies	Documented DVT during pregnancy or puerperium (not all objectively diagnosed)	FVL carriers: 7 DVT in association with 100 pregnancies FVL non-carriers: 1 DVT in association with 50 pregnancies	3.7 (0.4-170)*
572	Objectively diagnosed DVT during pregnancy or puerperium	0 DVT among primigravid cohort // 4/33 (9.1%) of women with history of DVT were carriers of FVL vs. 13/540 (2.4%) of controls (homozygotes included)	- // 5.6 1.2-19.5)*
52 age-matched parous controls; 118 pregnancies	90% objectively diagnosed VTE (superficial thromboses included); during pregnancy or puerperium; previous VTE in 12	FVL homozygotes: 19 VTE (25 SVT) FVL non-carriers: 0 DVT (1 SVT)	-
182 controls; 221 pregnancies	Objectively diagnosed VTE during pregnancy or puerperium	FVL homozygotes: 3 women with DVT FVL non-carriers: 1 woman with DVT	OR 41.3 (4.1-420)
81	First documented DVT in pregnancy or puerperium	FVL heterozygotes: 6 DVT in association with 242 pregnancies FVL non-carriers: 1 DVT in association with 215 pregnancies (FVL homozygotes: 1 DVT in association with 14 pregnancies)	5.3 (0.6-43.9)
4,751 (White 1,505)	First objectively diagnosed symptomatic DVT during pregnancy or puerperium	FVL carriers: 0 DVT in association with pregnancy FVL non-carriers: 4 DVT in association with pregnancy (race not known)	-

5.7 Pre-eclampsia

Pre-eclampsia is an important cause of maternal and fetal morbidity complicating 2-7% of pregnancies [87]. Pre-eclampsia is one of the leading causes of maternal mortality [64,65]. In Finland, pre-eclampsia and eclampsia cause about 12% of maternal deaths [67].

Pre-eclampsia is defined as high blood pressure after 20 weeks of gestation in a previously normotensive woman plus new-onset proteinuria. Definitions vary slightly among studies, but usually the ACOG criteria [88] are applied. Pre-eclampsia may be mild, just fulfilling the definition, or severe, including symptoms and findings like thrombocytopenia, elevated liver enzymes, epigastric or right upper-quadrant pain with nausea or vomiting, oliguria, cerebral symptoms, pulmonary edema, and seizures [87]. Pre-eclampsia is ultimately cured only by delivery, therefore often leading to preterm birth. Prematurity and fetal growth restriction, which is often associated with pre-eclampsia, affect the health of the newborn [87].

The etiology of this heterogeneous disease entity is still unknown [87,89]. Pre-eclampsia can be divided to placental pre-eclampsia originating from abnormal placental perfusion, and maternal pre-eclampsia originating from pre-existing problems in mother [90]. However, in an individual, pre-eclampsia may be caused by variable interaction of placental/fetal and maternal factors [89]. Factors that have been associated with an increased risk for pre-eclampsia include primigravidity, multifetal gestation, previous pre-eclampsia, obesity, pregestational diabetes, chronic hypertension or renal disease, family history of pre-eclampsia, and controversially thrombophilia [87]. Endothelial dysfunction is considered to be an important factor in its development [90,91]. Endothelial cell injury can lead to the activation of coagulation, vasoconstriction, reduced plasma volume due to "leaking endothelium", and glomerular capillary protein leak [91]. A thrombotic tendency may exacerbate the activation of coagulation.

5.7.1 FV Leiden and pre-eclampsia

Numerous studies with different designs have assessed association between pre-eclampsia and FV Leiden and many meta-analyses have tried to determine the true association.

In a meta-analysis by Dudding and Attia [92], the OR for association of FV Leiden with pre-eclampsia varied from 0.2-12.9 in 24 case-control studies. Studies were so heterogeneous that pooled OR was not calculated. In seven studies specifying severe pre-eclampsia, pooled OR was 3.0 (95% CI 2.0-4.7). These studies included 753 cases and 1,120 controls of women with reported ethnicity of Caucasian or Israeli. Heterozygous and homozygous carriers of FV Leiden were pooled.

In a meta-analysis by Lin and August [93], the combined OR for FV Leiden in 12 case-control studies assessing all pre-eclampsia was 1.8 (95% CI 1.1-2.9). Heterozygous and homozygous carriers were pooled. Statistical test for heterogeneity was significant (p=0.04) and a funnel plot analysis suggested publication bias (small negative studies missing). The studies included 1,798 cases and 1,471 controls of mostly Caucasian origin; in one study

the participants were Japanese and in one Australian study only 83% were Caucasian. In their meta-analysis of 11 case-control studies assessing severe pre-eclampsia, the pooled OR for FV Leiden was 2.2 (95% CI 1.3-3.9). Statistical test for heterogeneity was significant (p=0.009), but there were no suggestion of publication bias. These studies included 1,135 cases and 1,471 controls of mostly Caucasian origin; in three studies, 90-95% of women were Caucasian and in one study only 40% were Caucasian. As FV Leiden is mostly limited to the Caucasian population, inclusion of other ethnicities may influence the results.

In a systematic review and meta-analysis by Robertson *et al.* [79], heterozygous and homozygous carriers of FV Leiden were analyzed separately for the risk of pre-eclampsia. Fourteen studies assessing heterozygous FV Leiden had pooled OR of 2.2 (95% CI 1.5-3.3) with signs of heterogeneity (p=0.04). The studies included both mild and severe pre-eclampsia and study designs varied from retrospective case-control and cohort studies to one prospective cohort study. Studies included 1,951 cases and 1,971 controls, ethnicity was not specified. When five studies of severe pre-eclampsia were analyzed separately, the pooled OR for heterozygous FV Leiden was 2.0 (95% CI 1.2-3.4) without signs of heterogeneity. Five studies assessing homozygous FV Leiden had pooled OR of 1.9 (95% CI 0.4-7.9) without signs of heterogeneity. These studies included 612 cases and 536 controls, ethnicity was not specified.

In a recent systematic review and meta-analysis of nine prospective cohort studies assessing the association between FV Leiden and pre-eclampsia by Rodger *et al.* [3], the pooled OR was 1.23 (95% CI 0.89-1.70) indicating that FV Leiden is not associated with an increased risk for pre-eclampsia. There were no signs of heterogeneity. The meta-analysis comprised 21,833 unselected prospectively enrolled women with a spontaneous singleton pregnancy from Ireland, Israel, the United States, the United Kingdom, Australia, Greece, Sweden, and Canada. FV Leiden carriers had a 3.8% absolute risk for pre-eclampsia whereas in FV Leiden non-carriers the absolute risk was 3.2%. The prevalence of FV Leiden in these populations varied from 2.7% to 10.9%, ethnicity of participants was not specified.

Only two population-based studies of at least predominantly Caucasian study populations assess FV Leiden as a risk factor for pre-eclampsia. Both are retrospective registry-based cohort studies of geographically well-defined area, one from Scotland [94] (included in meta-analyses by Dudding, Lin, and Robertson [79,92,93]), and one from Norway [95] (included in meta-analysis by Dudding [96]). Both studies pooled heterozygotes and homozygotes in their analyses. The study from Scotland analyzed 494 pre-eclampsia cases and 163 controls, ethnicity was not reported. In this study, FV Leiden was not associated with an increased risk for pre-eclampsia (OR 0.9, 95% CI 0.4-2.1) [94]. The study from Norway analyzed 14,393 pregnancies of 5,874 women, ethnicity was not reported. In this study, FV Leiden was associated with an increased risk for pre-eclampsia (OR 1.6, 95% CI 1.2-2.3). As the study analyzed pregnancies, not women, several pregnancies for each women were included [95].

In a study by Dudding *et al.* [96], FV Leiden was not significantly associated with an increased risk for verified pre-eclampsia in a cohort of pregnant women (FV Leiden in 17/243 cases and 204/4,206 controls, OR 1.19, 95% CI 0.64-2.23). However, when they combined their study with five other cohort studies in meta-analysis to increase power, association between FV Leiden and pre-eclampsia

became significant (pooled OR 1.49, 95% CI 1.13-1.96). The pooled analysis included 860 cases and 18,340 controls from the United Kingdom, Sweden, the United States (two studies), Norway, and Ireland.

Meta-analyses described above include partly the same studies. A recent Canadian study by Kahn *et al.* [97] has not been included in any of them. This nested case-control study within a prospective cohort of pregnant women consisted of 113 pre-eclampsia cases and 443 controls. The study included different ethnicities, which were not specified. FV Leiden was not associated with an increased risk for pre-eclampsia (OR 1.1, 95% CI 0.4-2.7). The study showed that histopathologic features consistent with placental underperfusion were more common in cases than in controls. However, FV Leiden was not associated with these features.

Taken together, individual studies and meta-analyses assessing the association between FV Leiden and pre-eclampsia have yielded very conflicting results. The association, if any, seems to be modest.

5.8 Stillbirth

Although stillbirth is a rare pregnancy complication in developed countries, it causes strong emotional burden for the particular family. The stillbirth rate has been estimated to be 4.2-6.8 per 1,000 deliveries in developed countries [98]. In Finland, like in other Nordic countries, the stillbirth rate is even less and among the smallest in the world [99]. However, due to different definitions the stillbirth rate is difficult to compare between countries.

The precise definition of stillbirth varies in different countries and in different studies. The definition is based on gestational age of the fetus at the time of stillbirth (usually ≥20-24 weeks) or on the fetal weight (usually ≥500g) [99-101]. In Finland, stillbirth is defined as stillbirth at or after 22 weeks of gestation, or fetal weight ≥ 500 g [99]. Stillbirths are subclassified as early stillbirths at or before 28 weeks of gestation and late stillbirths after 28 weeks of gestation [101].

Risk factors for stillbirth include multiple pregnancy, nulliparity, advanced maternal age, pre-pregnant obesity, smoking, maternal diseases, previous stillbirth, and low socio-economic status [101,102]. The causes of stillbirth include maternal infections, placental lesions like *abruptio placentae*, or major infarction of the placenta, umbilical cord complications like prolapse, strangulation, or knot, and congenital anomalies [101,102]. However, 25-60% of stillbirths remain unexplained [101,102]. Thrombophilia has been hypothesized as one possible risk factor for stillbirth [100-102].

5.8.1 FV Leiden and stillbirth

Studies assessing the association between FV Leiden and stillbirths vary in many respects. Study designs, selection of cases and controls, definition of stillbirth, reporting of ethnicity, inclusion of women with previous thromboembolism, and inclusion of homozygotes in analyses differ. Case-control and cohort studies are summarized in tables 3 and 4.

In case-control studies (table 3), the odds ratio for association between FV Leiden and stillbirth varied from 0.7 to 9.2. In six of the nine studies, the association was statistically significant. Only two of the studies assessing unexplained stillbirth reported the study population to be Caucasian [103,104].

In retrospective cohort studies (table 4), the odds ratio varied from 1.3 to 4.4. In only one of these studies, the association was statistically significant (OR 2.2, 95% CI 1.5-3.4), but there the studied unit was not a woman but pregnancy [95]. In the only prospective cohort study to this date [105] (table 4), FV Leiden was associated with almost a 9-fold risk for stillbirth (OR 8.85, 95% CI 1.6-48.9). Cohort consisted of 1,707 nulliparous healthy women with a singleton pregnancy and heterogeneous ethnic background. However, there were only six stillbirths in the cohort.

In a meta-analysis by Rey *et al.* in 2003 [106], the pooled OR for association between FV Leiden and fetal loss after 19 weeks of gestation was 3.3 (95% CI 1.8-5.8). Analysis included six retrospective studies with no signs of heterogeneity (372 cases, 1,888 controls). In a meta-analysis by Dudding *et al.* in 2004 [92], the pooled OR for FV Leiden was 2.8 (95% CI 1.3-6.2) when assessing only isolated third trimester fetal losses. There were no signs of heterogeneity in this *post hoc* subanalysis of five studies. In a systematic review and meta-analysis by Robertson *et al.* in 2006 [79], the pooled OR for association between heterozygous FV Leiden and late fetal loss (third trimester) was 2.06 (95% CI 1.1-3.9) with no signs of heterogeneity. Analysis included six retrospective case-control and cohort studies with 151 cases and 1,503 controls, ethnicities were not reported. In a recent review by Werner *et al.* [100], a meta-analysis of eleven heterogeneous studies yielded a pooled OR of 3.6 (95% CI 2.1-6.2). The above four meta-analyses included partly same studies.

Taken together, the results of individual studies are partly conflicting, perhaps resulting from heterogeneity of the studies. In meta-analyses, FV Leiden has been associated with quite a constant 2-fold to 3-fold increased risk. Population-based studies are few.

Table 3. *FV Leiden and risk for stillbirth. Case-control studies.*

Study	Country	Self-reported study design	Study population	Cases
Gris *et al.* 1999 [107]	France	Case-control	Women without history of thromboembolism, miscarriage, PIH, or infection during pregnancy; ethnicity not reported	232
Kupferminc *et al.* 1999 [108]	Israel	Case-control	Jewish Ashkenazi, non-Ashkenazi, or mixed Ashkenazi	12
Martinelli *et al.* 2000 [103]	Italy	Case-control	White women ≤35 years without history of venous thrombosis	67
Many *et al*. 2002 [109]	Israel	Case-control	Ashkenazi and non-Ashkenazi	40
Weiner *et al*. 2004 [110]	Israel	Case-control	Jewish and Arabs	53
Gonen *et al.* 2005 [111]	Israel	Case-control	Different ethnic groups in Israel (cases and controls matched for ethnicity)	37
Sottilotta *et al.* 2006 [104]	Italy	Case-control	Caucasian women	47 (referred to thrombosis center for investigation)
Kocher *et al.* 2007 [112]	USA	Case-control	White (from a cohort of 4872 women with live birth)	32 (women with previously documented or self-reported stillbirth)
Simchen *et al.* 2010 [113]	Israel	Prospective cohort study (Case-control)	Ethnicity not reported	67 women (33 with "placental stillbirth"; 48% nulliparous)

* Calculated from the data given in the article (StatsDirect).

Abbreviations: PIH, pregnancy induced hypertonia

Controls	Stillbirth definition	Un-explained	Prevalence of FVL	OR (95% CI)
464 (matched for age and parity)	>22	Yes	Cases: 15/232, 6.5% Controls: 7/464, 1.5% (all heterozygous)	4.8 (1.8-12.4)
110 parous women without thromboembolic complications; matched for age and family origin	>23	Yes	Cases: 3/12, 25% Controls: 7/110, 6% (homozygotes included)	4.9 (1.1-22)
232	≥20 gestational weeks	Yes	Cases: 5/67, 7% Controls: 6/232, 3% (all heterozygous)	3.2 (1.0-10.9)
80 healthy parous women matched for age and ethnicity	≥27 gestational weeks	Yes	Cases: 3/40, 7.5% Controls: 3/80, 3.8% (all heterozygous)	1.5 (0.7-3.6)
59 parous women without thromboembolic complications	>24 gestational weeks	Yes	Cases: 9/53, 17% Controls: 5/59, 8.5% (homozygotes included)	2.2 (0.6-9.0)*
46 parous women without history of stillbirth, recurrent fetal loss or thromboembolism	27-42 gestational weeks	Yes	Cases : 4/37, 10.8% Controls: 7/46, 15.2% (homozygotes included)	0.68 (0.1-3.0)
217 (healthy parous women without pregnancy complications)	>20 gestational weeks	Yes	Cases: 11/47, 23.4% Controls: 7/217, 3.2%	9.2* (3.0-29.5)
96 (matched for age and gravidity)	Birth weight of fetus ≥500g	No	Cases: 6/32, 19% Controls: 2/96, 2%	10.9 (2.1-57)
637 low-risk nulliparous pregnant women from another study, same population	>20 gestational weeks	No	Cases: 16/67, 23.9% Controls: 24/637, 3.8%	8.0 (4.0-16.0)

Table 4. *FV Leiden and risk for stillbirth. Cohort studies.*

Study	Country	Self-reported study design	Study population	Carriers of FVL
Preston *et al.* 1996 [113]	UK, The Netherlands, Sweden, France, Spain, Italy, Austria, Germany, Israel	Cross-sectional multicenter (cohort) study	Parous women of EPCOT study; ethnicity not reported	141
Meinardi, *et al.* 1999 [114]	The Netherlands	Retrospective (family) cohort study	Thrombophilic families; propositi and relatives included; White parous women	228 (homozygotes included)
Tormene *et al.* 1999 [115]	Italy	Retrospective (family) cohort study	Parous family members of probands with VTE; ethnicity not reported	65 (homozygotes included)
Pabinger *et al.* 2000 [85]	Austria, Hungary, Germany	Multicenter retrospective cross-sectional study	Ethnicity not reported	64 homozygotes; 212 pregnancies
Baré *et al.* 2000 [116]	Hungary	(Retrospective) cohort study	Ethnicity not reported	128 (4 homozygotes included)
Völzke *et al.* 2003 [117]	Germany	Population-based cross-sectional study	1,768 parous women; ethnicity not reported	111 (homozygotes included)
Nurk *et al.* 2006 [94]	Norway	Retrospective (population-based) cohort study	5,874 women with 14,474 pregnancies; ethnicity not reported	1,049 pregnancies (5451 women) (homozygotes included)
Said *et al.* 2010 [104]	Australia	Prospective antenatal cohort study; "convenience sample"	1,707 healthy nulliparous women enrolled <22 gestational weeks; singleton pregnancies; no history of thrombosis or thrombophilia; heterogenic ethnicity	93 (homozygotes included)

* Calculated from the data given in the article (StatsDirect).

\# Numbers from meta-analysis by Rodger *et al*. [3].

Abbreviations: EPCOT, European Prospective Cohort on Thrombophilia

Non-carriers of FVL	Stillbirth definition	Un-explained	Stillbirth	OR (95% CI)
395	>28 gestational weeks	No	FVL carriers: 5/410, 1.2% pregnancies FVL non-carriers: 6/1,019, 0.6% pregnancies	2.0 (0.5-7.7) (adjusted for number of pregnancies and center)
121	>20 gestational weeks	No	FVL carriers: 13/228, 5.7% FVL non-carriers: 6/121, 5.0%	1.3 (0.5-3.7)
44	>24 gestational weeks; Verified from medical records	Yes	FVL carriers: 7/65, 10.8% FVL non-carriers: 1/44, 2.3%	4.4 (0.5-35.6)
52 age-matched parous controls; 118 pregnancies; FVL status unknown	>h23 gestational weeks (self -reported)	No	FVL carriers: 7/64, 11% Controls: 2/52, 4%	2.0 (0.4-9.7)
461	Self-reported intrauterine death	No	FVL carriers: 1 intrauterine death FVL non-carriers: 2 intrauterine deaths	1.8 (0.03-34.9)*
1,657	>h28 (self-reported)	No	FVL carriers: 7/111, 6.3% FVL non-carriers: 66/1,657, 4.0%	1.57 (0.8-3.25)
13,344 (423 women)	Not defined (mean gestational age 29.7 weeks)	No (registry data)	FVL Carriers: 26/1,049, 2.5% of pregnancies FVL non-carriers: 151/13,344, 1.1% of pregnancies	2.2 (1.45-3.4)
1,614	≥20 gestational weeks or birth weight of fetus ≥400g	Yes	FVL carriers: 2/93, 2.2% FVL non-carriers: 4/1,633, 0.2% #	8.85 (1.6-48.9)

5.9 Preterm birth

Preterm birth (birth before 37 completed weeks of gestation), occurring in 5-13% of deliveries in developed countries, is a major cause of neonatal morbidity and mortality [119,120]. In Finland, preterm delivery occurs in about 5% of deliveries [99].

Preterm birth is a heterogeneous clinical entity. Many pregnancy complications may lead to it, but in about half of the cases the cause of preterm birth remains unknown [120]. Preterm birth can be categorized to 1) spontaneous preterm birth due to a) onset of preterm labour or b) preterm premature rupture of membranes (PPROM), and to 2) indicated preterm birth including a) induced labour and b) cesarean section performed for maternal or fetal reasons [121]. Preterm birth can also be categorized by gestational age to extremely preterm (<28 gestational weeks), severely preterm (28-31 gestational weeks), and late preterm birth (32-36 gestational weeks) [121]. Preterm births at 32-36 gestational weeks have also been sub-classified to moderate prematurity (32-33 gestational weeks), and to near term (34-36 gestational weeks) [119]. However, as infants born at 34-36 gestational weeks are immature and have a greater risk of morbidity and mortality than infants born at term, it is recommended to refer them as late-preterm infants [122]. As a result, late preterm birth has different definitions in the literature. The etiological causes of preterm birth are partly different during different phases of pregnancy [119].

Inflammatory mechanisms have a key role in the initiation of normal labour [5]. Mechanisms that are thought to be involved in the initiation of preterm labour include infection or inflammation, uteroplacental ischemia or hemorrhage, uterine overdistention, stress, and other immunologically mediated processes [119]. In addition, genetic factors and race contribute to preterm birth [119,120]. Factors that are associated with an increased risk for preterm birth include multiple gestation, uterine anomalies, maternal diseases, extremes of maternal age, low pre-pregnancy BMI, smoking, and low socioeconomic and educational status [119,120].

Theoretically, thrombophilia could influence the initiation of preterm labour via thrombosis in the placenta causing uteroplacental ischemia or oxidative stress, or by activating inflammatory mechanisms. Polymorphisms in genes involved in coagulation and inflammation, including factor V, have been associated with preterm birth [123,124].

5.9.1 FV Leiden and preterm birth

Only three heterogeneous reports have shown positive association between FV Leiden and preterm birth. One study compared 205 very low birth weight (VLBW) preterm infants with 205 term infants (OR for FV Leiden 2.1, 95% CI 1.01-4.4) [125]. One study observed increased prevalence of FV Leiden in 50 women with preterm birth compared with the population prevalence (18% vs. 6.3%) [126]. FV Leiden has also been associated with preterm birth with evidence of placental hemorrhage (OR 4.8, 95% CI 1.6-14.2) [127]. Other current studies have not found a significant association between FV Leiden and preterm birth [112,128-132]. Many of the studies are small or have other limitations. Study design, selection of cases and controls, ethnicity, exclusion criteria, and even definition of

preterm birth differ. Case-control and cohort studies are summarized in tables 5 and 6. At present, no prospective unselected cohort studies have been published.

Taken together, only a limited number of heterogeneous studies have assessed the association between FV Leiden and preterm birth, and with conflicting results.

Cervical insufficiency is one cause of preterm birth. In one study, FV Leiden was associated with a 4-fold risk for cervical insufficiency and subsequent preterm birth (OR 4.2, 95% CI 1.5-13.6) [133]. The authors speculated that increased thrombin production caused by FV Leiden could intensify activation of inflammatory processes in the cervix leading to cervical insufficiency.

5.10 Current recommendations for screening of inherited thrombophilia in association with pregnancy complications

Screening for thrombophilia has been under debate since the first findings of association between thrombophilia and placenta-mediated pregnancy complications. However, screening for a risk factor is indicated only if the result influences the treatment of the patient [7]. LMWH is increasingly used in women at increased risk for these complications [134]. However, with the current knowledge, prophylaxis with low molecular weight heparin (LMWH) is not routinely recommended in women with a prior placenta-mediated pregnancy complication (pregnancy loss, pre-eclampsia, IUGR, placental abruption), whether they have inherited thrombophilia or are unselected [3,7,134]. Further randomized controlled trials are urgently needed.

In the light of this, in current guidelines, screening for inherited thrombophilia, or FV Leiden, is not recommended in unselected women with placenta-mediated pregnancy complications [7,134]. However, screening for thrombophilia in women with personal or family history of venous thrombosis is considered reasonable as it may influence the timing and intensity of venous thrombosis prophylaxis during pregnancy and puerperium [7,134]

Table 5. *Leiden and risk for preterm birth. Case-control studies.*

Study	Country	Self-reported study design	Study population	Cases	Controls
Göpel *et al.* 1999 [125]	Germany	Case-control	White infants	205 preterm infants with very low birth weight	205 random healthy term singletons
Erhardt *et al.* 2000 [126]	Hungary	Observational	Caucasian	50 women	- (population prevalence as control)
Valdez *et al.* 2004 [128]	Mexico	Case-control	Mexican	86 nonconsecutive mestizo women	228 1) healthy adults, male/female ratio 1:1 2) parous women (ethnicity not reported)
Resch *et al.* 2004 [129]	Austria	Single-center case-control	Ethnicity not reported	35 consecutive mothers with preterm infant admitted to neonatal intensive care unit	54 mothers with term infants in the same neonatal ward (>37 gestational weeks)
Härtel *et al.* 2005 [130]	Germany	Prospective multicenter case-control	Mainly White	397 mothers of preterm very low birth weight singletons	278 mothers of term singletons
Kocher *et al.* 2007 [112]	USA	Case-control	White	99 women	294 women matched for gravidity and age
Uvuz *et al.* 2009 [131]	Turkey	Case-control	Ethnicity not reported	50 women	50 healthy women with uncomplicated pregnancy and term labour
Kramer *et al.* 2009 [132]	Canada	Nested case-control study in prospective multicenter cohort	Information on race or ethnic origin was not collected	206 women	444 women with term delivery (for each case two next delivering women in the same hospital)

* Calculated from the data given in the article (StatsDirect).

Table 6. *FV Leiden and risk for preterm birth. Cohort studies.*

Study	Country	Self-reported study design	Study population	Carriers of FVL	Non-carriers of FVL
Gargano *et al.* 2009 [127]	USA	Prospective multicenter cohort study, subcohort sample	White (women with unexplained high MSAFP were oversampled; 7% of the cohort)	34 women Subanalysis: 27 women	526 women Subanalysis: 409 women

Preterm birth definition	Exclusion criteria	Prevalence of FVL	OR (95% CI)
<37 gestational weeks and birth weight <1,500 g (VLBW)	-	Cases: 10.7% Controls: 5.4% (carrier status not specified)	2.1 (1.01-4.4)
<37 gestational weeks	-	Cases: 9/50, 18.0% Population: 6.3% (Cases heterozygous)	3.5*
22-36 gestational weeks	Induced delivery	Cases: 2/86, 2% Controls: 11/228, 5% (all heterozygous)	0.5 (0.05-2.2)*
≤35 gestational weeks	Chronic illness, infection, drug abuse, bicornate uterus, incompetent cervix, multiple gestation, erythroblastosis, non-immune hydrops, PROM, polyhydramnion, iatrogenic	Cases: 3/35, 8.6% Controls: 3/54, 5.6% (all hetegozygous)	1.6 (0.3-8.4)
<37 gestational weeks and birth weight <1500 g (VLBW)	Lethal disability	Cases: 8.3% Controls: 5.2% (homozygotes included)	1.7 (0.9-3.5)*
<37 gestational weeks	Trauma, incompetent cervix, thalassemia, vWD, antiphospholipid antibodies, age >45	Cases: 8/99, 8.1% Controls: 24/294, 8.2% (carrier status not specified)	0.99 (0.4-2.3)
28-36 gestational weeks	PROM, chronic hypertension, diabetes mellitus, liver or renal disease, fetal anomaly, multiple birth, oligo- or polyhydramnion, uterine anomaly	Cases: 5/50, 10.0% Controls: 6/50, 12.0% (homozygotes included)	0.7 (0.1-3.0)*
Spontaneous delivery <37 gestational weeks	Multiple pregnancy, age <18, severe chronic illness (other than asthma, hypertension or diabetes), placenta previa, history of incompetent cervix, major anomaly of fetus	Cases: 4.9% Controls: 4.3% (all heterozygous)	1.1 (0.5-2.5)

Preterm birth definition	Exclusion criteria	Preterm birth	OR (95% CI)
<37 gestational weeks Subanalysis: <37 gestational weeks and evidence of placental hemorrhage	Multiple pregnancy, pre-existing diabetes mellitus, age <15, congenital anomaly	FVL carriers: 12/34, 35% FVL non-carriers: 140/526, 27% Subanalysis: FVL carriers: 5/27, 18.5% FVL non-carriers: 23/409, 5.6% (homozygotes included)	Preterm birth: 1.7 (0.8-3.7) Subanalysis: 4.8 (1.6-14.2)

6 AIMS OF THE STUDY

The main aim of the study was to assess FV Leiden as a risk factor for pregnancy complications in which prothrombotic mechanisms may play a part. The specific aims were:

I

To assess the magnitude of the risk for venous thromboembolism during pregnancy and puerperium caused by FV Leiden. To assess the interaction between FV Leiden and other known risk factors for venous thrombosis. To estimate the absolute and attributable risks of FV Leiden on individual and population levels.

II

To assess FV Leiden as a risk factor for pre-eclampsia and eclampsia. To assess FV Leiden as a risk factor in subgroups of pre-eclampsia: severe pre-eclampsia, early pre-eclampsia, and pre-eclampsia with IUGR.

III

To assess FV Leiden as a risk factor for unexplained stillbirth. To assess early and late unexplained stillbirth separately.

IV

To assess FV Leiden as risk a factor for preterm birth. To assess FV Leiden as a risk factor in clinical subgroups of preterm birth: early and late preterm birth, and preterm birth without other pregnancy complications.

7 MATERIALS AND METHODS

7.1 Study design

The study design is a nested case-control study within a fixed cohort of 100,000 consecutive pregnant women in Finland. Study population is ethnically homogeneous and represents Finnish women from all over the country with pregnancies lasting beyond 8 to 12 weeks of gestation.

A nested case-control strategy is feasible when resources are scarce. It yields readily generalizable results and offers advantages over case-control designs, nested in an unknown cohort, to assess and ensure internal validity of the results. Also, it allows calculation of the same parameters as in true cohort studies.

In this study, selection bias was avoided by using the Hospital Discharge Register in identification of cases and controls. Accuracy of registry-based diagnoses was ensured by checking medical records of all participants. Strict diagnostic criteria were used for each studied disease entity. Information bias was minimized by collecting data from medical records and questionnaires on to standardized forms blinded for laboratory results.

7.2 Ethical considerations

The study was approved by the ethics committee of the Finnish Red Cross Blood Service (February 18th, 1997) and by the Ministry of Social Affairs and Health (July 16th, 1997, Dnro18/08/97). The Population Register Centre gave permission for address data on February 15th, 2000 (1273/40/99). All participants gave written informed consent. Every hospital gave permission to use medical record archives. The study was planned and conducted according to the Personal Data Act (523/1999), the Medical Research Act (488/1999) and the Medical Research Decree (986/1999).

7.3 Study population

7.3.1 Ethnicity

At the beginning of the study in 1997, Finland had a population of 5.15 million people with about 58,000 births per year. The Finnish population is ethnically very homogenous Caucasian. According to Statistics Finland (March 19th, 2010), in 1997 only 118,070 persons living in Finland were born outside Finland, and of these, only 34,476 were born outside Europe. Of these, 9,957 were women aged 15 to 49 years, i.e., 0.8% of the same-aged women in the population. Women were considered for the study if they had Finnish unique identification code, and if their mother tongue was Finnish or Swedish, which practically excludes all first generation migrants. Mother tongue was ascertained from the Population Register Centre from where also the addresses of the participants were obtained. In the European Union, processing of personal data revealing racial or ethnic origin is prohibited (Directive 95/46/EC of the European Parliament and of the Council of 24 October 1995 on the protection of individuals with regard to the processing of personal data and on the free movement of such data).

7.3.2 National Register of Blood Group and Blood Group Antibodies of Pregnant Women

In Finland, practically all pregnant women contact their local Maternity Welfare Clinic during 8th to 12th weeks of pregnancy. Attending antenatal care is a prerequisite for the benefits given by the Social Insurance Institution. At the first visit, samples are taken for blood group serology tests, which are performed in the Finnish Red Cross Blood Service at the department of antenatal serology. The department maintains the National Register of Blood Groups and Blood Group Antibodies of Pregnant Women from which data for 100,000 consecutive pregnant women were obtained. Only the first pregnancy of each woman after January 1st, 1997 (index pregnancy) was included in the cohort. The time scale of identification of 100,000 consecutive pregnant women was from January 1997 to October 1998.

7.3.3 National Hospital Discharge Register

The National Institute for Health and Welfare maintains the National Hospital Discharge Register with diagnoses classified according to the International Classification of Diseases (ICD-10 since 1996). Up to three diagnoses are registered for each inpatient care period. Personal unique identification codes were used to link the two registers to obtain diagnoses for the 100,000 consecutive pregnant women. ICD-10 codes retrieved from the Hospital Discharge register are shown in the appendix. As the pregnancies of women identified last ended in 1999, diagnoses from the Hospital Discharge Register were obtained from the years 1997-1999. When identification of pregnant women started, the new ICD-codes had been in use for one year.

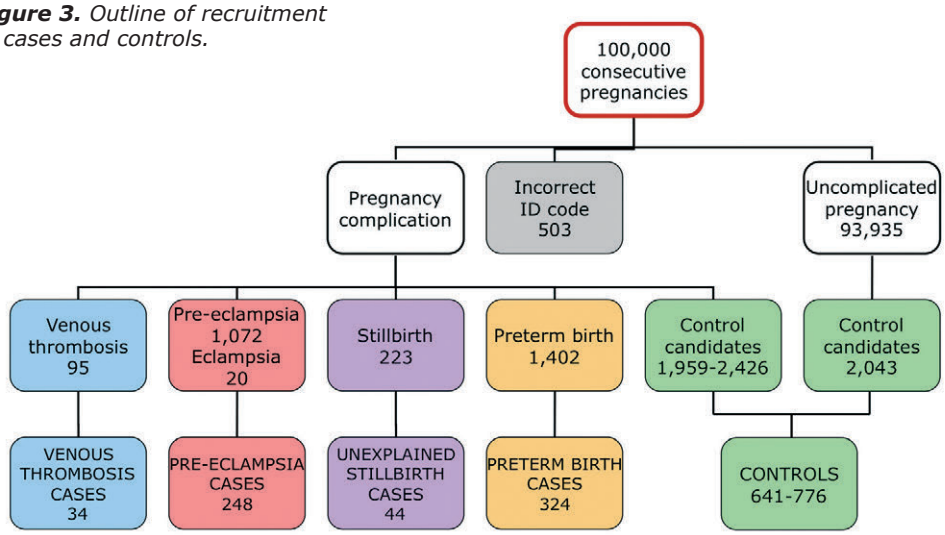
7.3.4 Recruitment of cases and controls

Figure 3 outlines the recruitment of cases and controls. In the cohort of 100,000 pregnant women, 503 women had an incorrect unique identification code and were excluded. Of the rest, 5,562 women had some pregnancy complication in the Hospital Discharge Register and 93,935 had none of these.

When planning the study, the aim was to get 500 cases with pre-eclampsia and preterm birth, and four controls per case. Because the participation rate was assumed to be about 75%, somewhat more case-candidates were invited to the study. All possible cases of pregnancy-associated venous thrombosis and stillbirth were invited. All eligible controls were planned to be used in all four sub-studies.

After identifying case-candidates and control-candidates, their addresses and mother tongue were obtained from the Population Register Centre. An invitation letter was sent to women who fulfilled the invitation criteria (alive, mother tongue Finnish or Swedish, residence in Finland). Women were informed about the study and they were asked to return a form with information whether they wanted to participate in the study or not. Those who did not respond were sent a reminder. Altogether 4,548 invitations and 1,832 reminders were sent, and 2,269 women participated (49.9%). Questionnaires and tubes for blood samples along with a form for informed consent were sent to those who wanted to participate. Participants gave blood samples for serological and DNA tests in a laboratory

Figure 3. *Outline of recruitment of cases and controls.*



of their choice or in the nearest regional blood centre. Blood samples, filled questionnaires, and informed consents were mailed to the Helsinki Blood Centre.

The medical records of participants were reviewed in 49 maternity hospitals in Finland to gather clinical data. All data were collected on standardized forms blinded to laboratory results.

7.4 Cases and controls

7.4.1 Study I – Pregnancy-associated venous thrombosis

Of the 100,000 consecutive pregnant women, 95 had an ICD-code for any venous thrombosis. Of those, 88 fulfilled the invitation criteria and 70 (80%) participated. In 34 women (cases) an objectively diagnosed deep venous thrombosis (DVT) was verified from medical records: 21 had distal DVT, 11 had proximal DVT, 1 had pulmonary embolism, and 1 had cerebral vein thrombosis. Of the 36 women excluded from the study, 12 had superficial venous thrombosis, 4 had non-objectively diagnosed thrombosis, and 20 had had venous thrombosis before or more than 10 weeks after the index the pregnancy.

Controls were sampled randomly (every 46th pregnant woman) from the 93,935 women without pregnancy complications in the Hospital Discharge Register. Of the 2,043 women, 1,930 fulfilled the invitation criteria and 843 (44%) participated. After review of medical records, 641 women were eligible as controls (no venous or arterial thrombosis, pre-eclampsia, or stillbirth in the index pregnancy or before, and no thrombosis prophylaxis, miscarriage, premature birth, or IUGR in the index pregnancy).

7.4.2 Study II – Pre-eclampsia

Of the 100,000 consecutive pregnant women, 1,084 had an ICD-10 code for pre-eclampsia (O14.0, O14.1, O14.9) or eclampsia (O15.0, O15.1, O15.2, O15.9). The first 685 consecutive women were picked as case candidates. Of them, 665 fulfilled the invitation criteria and 411 participated (62%). A review of their medical records revealed that 248 women (60%) fulfilled the strict diagnostic criteria for pre-eclampsia (cases).

The control group comprised two randomly selected subgroups. First, every 46th from the 93,935 women without pregnancy complications was selected as a control candidate. Of the 2,043 women, 1,930 fulfilled the invitation criteria and 843 participated (44%). After a review of medical records, 641 women were eligible as controls (no pregnancy complications in the index pregnancy and no history of hypertension, stillbirth, pre-eclampsia, or thrombosis). Second, from the 4,478 women with a pregnancy complication other than pre-eclampsia (stillbirth, miscarriage, premature birth, IUGR, or venous or arterial thrombosis), 1,965 women were picked as control candidates. Of them, 1,838 fulfilled the invitation criteria and 1,022 women participated (56%). Of them, every 24th was selected as a control to ascertain that the proportion of women with complications other than pre-eclampsia would be the same as in the population. A review of their medical records verified that 38 were eligible (no pre-eclampsia). Altogether, the control group comprised 679 women. See flow-chart in the Study II (Fig. 1).

7.4.3 Study III – Stillbirth

Of the 100,000 consecutive pregnant women, 224 had an ICD-10 code for stillbirth (O36.4). Of them, 222 fulfilled the invitation criteria and 120 participated (54%). A review of their medical records revealed that 44 women fulfilled the criteria for unexplained stillbirth (cases). Of the excluded women, 22 had intrauterine fetal deaths before gestational week 22. In two women the diagnosis was wrong and in two women relevant information was missing. Furthermore, 16 stillbirths due to lethal congenital developmental conditions, 23 stillbirths due to umbilical cord complications, and 14 stillbirths due to infections were excluded (three women had infection and umbilical cord complication). There were no birth injuries, isoimmunizations, or advanced stage twin-twin transfusion syndromes.

The control group comprised two randomly selected subgroups. First, every 46th woman of the 93,935 women without pregnancy complications was selected as a control candidate. Of the 2,043 control candidates, 1,930 fulfilled the invitation criteria and 843 participated (44%). After a review of medical records, 729 women were eligible as controls (no pregnancy complications in the index pregnancy or previous stillbirth). Second, of the 5,338 women with a pregnancy complication other than stillbirth, 2,426 were chosen as control candidates. Of them, 2,294 fulfilled the invitation criteria and 1,313 participated (57%). Of them, every 28th was randomly picked as a control to ascertain that the proportion of women with complications other than stillbirth would be the same as in the population. A review of their medical records verified that 47 were eligible (no history of stillbirth). Altogether, the control group comprised 776 controls. See flow-chart in the Study III (Fig. 1).

7.4.4 Study IV – Preterm birth

Of the 100,000 consecutive pregnant women, 1,402 had an ICD-code for preterm birth (ICD O60). The first 670 consecutive women were picked up as case-candidates. Of them, 623 fulfilled the invitation criteria and 331 participated (53%). A review of their medical records revealed that 324 women fulfilled the criteria for preterm birth (cases).

Control candidates were randomly sampled from the cohort of 100,000 women. First, every 46th woman of the 93,935 women without pregnancy complications (preterm birth, birth of a small-for-gestational-age fetus, pre-eclampsia, miscarriage, stillbirth, or arterial or venous thrombosis) in the index pregnancy was selected as a control candidate (2,043). Of them, 1,930 fulfilled the invitation criteria and 843 (44%) participated. A check of their medical records verified that 705 were eligible as controls (no pregnancy complications in the index pregnancy and no previous preterm birth). Second, the control group was supplemented to avoid selection bias. Of the 4,160 women with complications in the index pregnancy, 1,959 consecutive were picked as control candidates. Of them, 1,874 fulfilled the invitation criteria and 1,093 (58%) participated. Of those, every 23rd was randomly picked as a control (47). Supplementation ascertained that the proportion of pregnancy complications other than preterm birth, some possibly associated with FV Leiden, was the same in controls as in the cohort of 100,000 women. Altogether the control group comprised 752 women. See flow-chart in the Study IV (Fig. 1).

7.4.5 Population sample

A population sample of 644 first-time blood donors was genotyped to estimate the prevalence of the seven polymorphisms in the Finnish population.

7.5 Definitions

Venous thrombosis was defined as objectively diagnosed distal or proximal deep venous thrombosis (DVT), pulmonary embolism, or cerebral vein thrombosis during the index pregnancy or 10 weeks after delivery or termination of pregnancy. The verification of thrombosis was done by ultrasound, venography, magnetic resonance imaging, or computed tomography. Superficial venous thromboses were excluded. First-time and recurrent venous thromboses were considered as a manifestation of the same thrombotic disease. Cases with recurrent thrombosis were included but cases with first-time venous thrombosis were also analyzed separately.

Pre-eclampsia was defined as the systolic blood pressure ≥140 mmHg and the diastolic blood pressure ≥90 mmHg with new-onset proteinuria (repeatedly ≥0.3 g/l or ≥0.5 g/24 hours or dipstick ≥+ representing values ≥0.3 g/l) after 20 weeks of pregnancy in a previously normotensive woman. Eclampsia was defined as seizure associated with pre-eclampsia. Severe pre-eclampsia was defined as the systolic blood pressure >160 mmHg measured at least twice, the diastolic blood pressure >110 mmHg measured at least twice, proteinuria >3 g/

l, or proteinuria >5 g/24 hours. Eclampsia and pre-eclampsia complicated with severe physical symptoms (epigastric pain, visual symptoms, oliguria, dyspnea) were also classified as severe pre-eclampsia. Early pre-eclampsia was defined as the onset of pre-eclampsia before the gestational week 34. (Definitions slightly modified from the ACOG criteria [88].)

Unexplained stillbirth was defined as intrauterine fetal death at 22 weeks of gestation or later (according to the definition used in Finland [99]) excluding stillbirths due to lethal congenital developmental conditions (chromosome anomalies, malformations), umbilical cord complications (e.g. prolapse, true knot, strangulation), infections (e.g. chorionamnionitis, cytomegalovirus, parvovirus, *Listeria monocytogenes*), birth injuries, isoimmunizations, and advanced stage twin-twin transfusion syndromes. Placental infarction and placental abruption were not excluded as they could be etiologically related to the exposure and outcome. Late stillbirth was defined as intrauterine fetal death at 28 weeks of gestation or later.

Preterm birth was defined as birth at or after 22 and before 37 completed weeks of gestation (according to the definition used in Finland [99]). Preterm birth was defined early when it occurred before 32 weeks, and late when it occurred at or after 32 weeks of gestation. Although the exact determination of gestational age is difficult, the best clinical estimate from the medical records was used in this study.

Intrauterine growth restriction (IUGR) was defined as the child's birth weight being minus two standard deviations or less for gestational age. Birth weight standards based on the Finnish population, separate for girls and boys, were available from the gestational day 195 onwards [135].

Maternal age was the age at the first visit at to the antenatal care.

Body mass index (BMI) was defined as pre-pregnancy weight in kilograms divided by the square of height in meters (kg/m^2). Pre-pregnancy weight and height were collected from medical records and confirmed from questionnaires.

7.6 Laboratory methods

Genomic DNA was isolated from blood samples collected in EDTA tubes by using a commercial kit (QIAamp® DNA Blood Mini Kit, Qiagen, Hilden, Germany). Subjects were genotyped for seven polymorphisms in the Finnish Genome Center. Genotyping method was based on restriction enzyme digestions after PCR. Assays were performed multiplexed in a total volume of 5 µl in the conditions described elsewhere [136,137]. A fluorescent label (6-FAM, NED, or HEX) was included in the primer. An additional restriction site served as an internal control for the digestion reaction. If a natural digestion site was lacking, primer-induced restriction analysis was used. PCR products were digested with 1U of specific restriction enzyme (New England Biolabs, Beverly, MA, USA) and then separated with the ABI PRISM 377 (PE Applied Biosystems, Foster City, CA, USA)

DNA sequencer. The data were analyzed using Genescan 3.1 and Genotyper 2.5 software (PE Applied Biosystems). Samples with a missing genotype result for FV Leiden or FII G20210A were analyzed with another method (Factor V Leiden Kit and Factor II (Prothrombin) G20210A Kit with LightCycler® Instrument, Roche Diagnostics GmbH, Mannheim, Germany) in Finnish Red Cross Blood Service. In addition, positive findings of FV Leiden and FII G20210A mutations were confirmed with the latter method. All results were concordant and all markers obeyed the Hardy-Weinberg equilibrium.

7.7 Statistical analysis

To test differences between cases and controls, Pearson Chi-Square or Fisher's Exact Test were used for discrete variables. Logistic regression was used to estimate crude and adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) for dummy variables.

SPSS® version 12.0.1 (SPSS Inc. Chicago, Illinois, USA) in Studies I-II and SPSS® version 15.0.1 in Studies III-IV (IBM Corporation, Somers, NY, USA), and StatsDirect statistical software version 2.7.3 (StatsDirect Ltd, Cheshire, UK) were used for statistical analyses. A two-sided p-value of <0.05 was considered statistically significant.

When appropriate, possible confounding was controlled by restriction and stratification. Effect modification was explored by observing strata. To control for the possible confounding effect of gravidity, primigravid women were studied separately in Studies II and IV. To control for the possible effect of multiple pregnancy, women with a singleton pregnancy were studied separately in Studies III and IV. To control for the heterogeneous pathogenesis of preterm birth in Study IV, a subgroup analysis was done of women (cases and controls) with pregnancies lasting ≥195 gestational days without stillbirth, pre-eclampsia, IUGR, placental abruption, or chorionamnionitis.

Specifically in Study I, attributable risk (AR) was calculated as follows: $AR = I_e - I_0$ [138]. In this context, I_e is the incidence of venous thrombosis in FV Leiden carriers and I_0 the incidence of venous thrombosis in non-carriers. AR indicates the risk for pregnancy-associated venous thrombosis among FV Leiden carriers attributable to the FVL. Attributable risk proportion (AR%) was calculated using formula $AR\% = (AR / I_e) \times 100$ [138]. AR% states what proportion of pregnancy-associated venous thrombosis among FV Leiden carriers can be attributed to the FVL. Population attributable risk (PAR) was calculated as follows: $PAR = I_T - I_0$ [138]. In this context, I_T is the incidence rate of venous thrombosis in the whole studied cohort. PAR indicates the risk for pregnancy-associated venous thrombosis attributable to the FV Leiden in the population of women with pregnancies not ending during the first trimester. Population attributable risk proportion (PAR%) was calculated using formula $PAR\% = (PAR / I_T) \times 100$ [138]. PAR% states the proportion of pregnancy-associated venous thrombosis in the population attributable to the FVL.

8 RESULTS

Table 7 summarizes the genotype frequencies of FV Leiden in cases and controls in Studies I-IV and presents the overall risk associated with FV Leiden and the studied disease entity.

Table 7. *Genotype frequencies of FV Leiden in Studies I-IV.*

Genotype #		Number of Cases (%)	Number of controls (%)	Odds ratio (95% CI)*
I Venous thrombosis		**34**	**641**	
FV Leiden	GG	27	627	11.6 (3.6-33.6)
	GA	6 (17.6)	14 (2.2)	
	AA	1 (2.9)	0	
II Pre-eclampsia		**248**	**679**	
FV Leiden	GG	238	663	1.7 (0.8-3.9)
	GA	10 (4.0)	16 (2.4)	
	AA	0	0	
III Stillbirth		**44**	**776**	
FV Leiden	GG	40	756	3.8 (1.2-11.6)
	GA	4 (9.1)	20 (2.6)	
	AA	0	0	
IV Preterm birth		**324**	**752**	
FV Leiden	GG	305	733	2.4 (1.3-4.6)
	GA	19 (5.9)	19 (2.5)	
	AA	0	0	

Abbreviations: CI, confidence interval

*Carriers of the FV Leiden (heterozygotes and homozygotes) versus wild-type.

Genotype: GG, wild-type homozygote; GA, FV Leiden heterozygote; AA, FV Leiden homozygote.

The prevalence of FV Leiden was 2.2-2.6% in controls. Excluding the first study, controls in Studies II-IV included also women with prior venous thromboses and women with other pregnancy complications. In the population sample of 644 blood donors, the prevalence of FV Leiden was 2.4%.

In Study I, the prevalence of FV Leiden was unequal in older and younger controls. The prevalence of FV Leiden was 7.7% (6/78) in controls over the age of 35 and 1.4% (8/563) in controls under the age of 35 (p=0.009). The result was the same when not only eligible but all control candidates (843) were considered (8.7% vs. 2.3%, p=0.002)). Neither parity nor gravidity affected the finding.

8.1 Study I - Pregnancy-associated venous thrombosis

Assessing index pregnancies, FV Leiden was associated with an 11-fold increased risk for pregnancy-associated venous thrombosis (OR 11.6, 95% CI 3.6-33.6). When analyzing only cases whose first venous thrombosis occurred in association with the index pregnancy, FV Leiden was associated with a 6-fold risk (OR 5.8, 95% CI 1.6-21.8). When only primigravid cases were analyzed, FV Leiden was associated with a 9-fold risk (OR 8.7, 95% CI 1.5-50.6).

Table 8. *Risk associated with FV Leiden in pregnancy-related venous thrombosis.*

	FV Leiden, n (%)	Odds ratio (95% CI)
All women		
Controls, n=641	14 (2.2)	1.0 *
Venous thrombosis, n=34	7 (20.6)	11.6 (3.6-33.6)
First venous thrombosis, n=26	3 (11.5)	5.8 (1.6-21.8)
Primigravid women		
Controls, n=223	5 (2.2)	1.0 *
Venous thrombosis, n=12	2 (16.7)	8.7 (1.5-50.6)

Abbreviations: CI, confidence interval.

*Reference group.

The risk associated with FV Leiden was modified by blood group, BMI, and age. The risk for pregnancy-associated venous thrombosis was 25-fold (OR 25.1, 95% CI 6.6-95.4) in women who had FV Leiden and non-O blood group, compared with women without FV Leiden and with blood group O. In women with FV Leiden and BMI over 30, the risk was 75-fold (OR 75.5, 95 % CI 6.7-846.5, adjusted for age), compared with women without the mutation and BMI less than 25. In women with FV Leiden and age over 35, the risk was nearly 60-fold (OR 58.1, 95% CI 4.2-799.7, adjusted for BMI), compared with women without the mutation and age less than 25. (See table 4 in Study I.)

In carriers of FV Leiden, 92% (95% CI 80-96) of thromboses were attributable to this mutation (AR%). In the whole study population, 19% (95% CI 16-20) of thromboses were attributable to FV Leiden (PAR%). (See table 5 in Study I.)

8.2 Study II - Pre-eclampsia

As different risk factors may be associated with different clinical entities of pre-eclampsia, subgroups were analysed separately. In addition to pre-eclampsia as a whole (248 cases), severe pre-eclampsia (168 cases, 68%), early pre-eclampsia (77 cases, 31%), and pre-eclampsia with IUGR (53 cases, 21%) were analyzed separately. Severe pre-eclampsia included 12 cases with eclampsia. Subgroups were partly overlapping. Pre-eclampsia was recurrent in 18% of multigravid cases.

As pre-eclampsia is particularly a disease of the first pregnancy, primigravid subjects were analyzed separately. Of the 143 primigravid cases, 99 (69%) had severe pre-eclampsia, 44 (31%) had early pre-eclampsia, and 29 (20%) had pre-eclampsia with IUGR. Subgroups were partly overlapping.

Table 9 shows the association of FV Leiden with subgroups of pre-eclampsia. The point estimates of the risk were 1.5-2.5 when all women were analyzed and 2.4-3.4 when primigravid women were considered. However, these associations were not statistically significant.

Table 9. *Risk associated with FV Leiden in pre-eclampsia and its subgroups.*

	FV Leiden, n (%)	Odds ratio (95% CI)
All women		
Controls, n=679	16 (2.4)	1.0 *
Pre-eclampsia, n=248	10 (4.0)	1.7 (0.8-3.9)
Severe pre-eclampsia, n=168	6 (3.6)	1.5 (0.6-4.0)
Early pre-eclampsia, n=77	4 (5.2)	2.3 (0.7-7.0)
Pre-eclampsia and IUGR, n=53	3 (5.7)	2.5 (0.7-8.8)
Primigravid women		
Controls, n=232	5 (2.2)	1.0 *
Pre-eclampsia, n=143	8 (5.6)	2.7 (0.9-8.4)
Severe pre-eclampsia, n=99	5 (5.1)	2.4 (0.7-8.5)
Early pre-eclampsia, n=44	3 (6.8)	3.3 (0.8-14.4)
Pre-eclampsia and IUGR, n=29	2 (6.9)	3.4 (0.6-18.2)

Abbreviations: CI, confidence interval; IUGR, intrauterine growth restriction.
*Reference group.

8.3 Study III – Stillbirth

Of the unexplained stillbirths in 44 cases, 12 (27%) occurred during 22-27 weeks of gestation (early) and 32 (73%) occurred at or after 28 weeks of gestation (late). The frequency of FV Leiden did not differ in these two groups (early 1/12, 8% vs. late 3/32, 9%). Late unexplained stillbirth and unexplained stillbirth with placental lesions (placental infarction or placental abruption) were analyzed separately. As twin pregnancy is a major risk factor for stillbirth, women with a singleton pregnancy were analyzed separately. Stillbirth was recurrent in one case. Of the primigravid women, none of the 13 cases and 7 of the 265 controls (2.6%) were carriers of FV Leiden.

Table 10 shows the association of FV Leiden with unexplained stillbirth. FV Leiden was associated with a 3-fold to 4-fold risk, whether analyzing all unexplained stillbirths or late unexplained stillbirths in all pregnancies or in singleton pregnancies. The association seemed to be pronounced when assessing unexplained stillbirth with placental lesions.

Table 10. *Risk associated with FV Leiden in subgroups of stillbirths.*

	FV Leiden, n (%)	Odds ratio (95% CI)
All pregnancies		
Controls, n=776	20 (2.6)	1.0 *
Unexplained stillbirth #, n=44	4 (9.1)	3.8 (1.2-11.6)
Late unexplained stillbirth # (≥ 28 weeks of gestation), n=32	3 (9.4)	3.9 (1.1-13.9)
Unexplained stillbirth # with placental lesions ¤, n=9	2 (22.2)	10.8 (2.1-55.3)
Singleton pregnancies		
Controls, n=762	20 (2.6)	1.0 *
Unexplained stillbirth #, n=39	3 (7.7)	3.1 (0.9-10.9)
Late unexplained stillbirth # (≥ 28 weeks of gestation), n=29	3 (10.3)	4.3 (1.2-15.3)
Unexplained stillbirth # with placental lesions ¤, n=9	2 (22.2)	10.6 (2.1-54.3)

Abbreviations: CI, confidence interval.

* Reference group.

Chromosome anomalies, malformations, infections, and umbilical cord complications excluded.

¤ Placental infarction or placental abruption.

8.4 Study IV – Preterm birth

Of the 324 cases, 78 (24%) had birth before 32 weeks of pregnancy (early preterm birth) and 246 (76%) had birth at 32-36 weeks of pregnancy (late preterm birth). Preterm birth was recurrent in 22% of multigravid cases. As pathophysiology of early and late preterm birth may differ, they were analyzed also separately.

FV Leiden was significantly associated with preterm birth (OR 2.4) and especially with late preterm birth (OR 2.9), but not with early preterm birth (OR 1.0). The association was significant also when primigravid cases and controls were analyzed (OR 3.3) and when cases and controls without stillbirth, pre-eclampsia, IUGR, placental abruption, or chorionamnionitis were analyzed (OR 2.6). Results are summarized in table 11.

FV Leiden was associated with preterm birth whether there had been premature rupture of membranes or not (OR 2.8, 95% CI 1.01-7.7; and OR 2.2, 95% CI 1.1-4.6) and when indicated deliveries (induced labour or cesarean delivery) were excluded from cases and controls (OR 3.6, 95% CI 1.7-7.8).

As a twin pregnancy predisposes to preterm birth, singleton pregnancies were analyzed separately. The results for singleton pregnancies were similar to the results for all pregnancies (table 11).

When primigravid women with singleton pregnancies without stillbirth, pre-eclampsia, IUGR, placental abruption, or chorionamnionitis were analyzed, FV Leiden was associated with nearly a 5-fold risk for late preterm birth (OR 4.7, 95% CI 1.5-15.3).

Table 11. *Association of FV Leiden with preterm birth in subgroups of cases and controls.*

	FV Leiden, n (%)	Odds ratio (95% CI)
All pregnancies		
Controls, n=752	19 (2.5)	1.0 *
Preterm birth, all, n=324	19 (5.9)	2.4 (1.3-4.6)
Early preterm birth (<32 weeks of gestation), n=78	2 (2.6)	1.0 (0.2-4.4)
Late preterm birth (32-36 weeks of gestation), n=246	17 (6.9)	2.9 (1.5-5.6)
Primigravid controls, n=262	6 (2.3)	1.0 *
Primigravid cases, n=112	8 (7.1)	3.3 (1.1-9.7)
Controls without complications #, n=705	17 (2.4)	1.0 *
Preterm birth without other complications #, n=229	14 (6.1)	2.6 (1.3-5.4)
Singleton pregnancies		
Controls, n=742	19 (2.6)	1.0 *
Preterm, all, n=279	18 (6.5)	2.6 (1.4-5.1)
Early preterm birth (<32 weeks of gestation), n=69	2 (2.9)	1.1 (0.3-5.0)
Late preterm birth (32-36 weeks of gestation), n=210	16 (7.6)	3.1 (1.6-6.2)
Primigravid controls, n=261	6 (2.3)	1.0 *
Primigravid cases, n=91	7 (7.7)	3.5 (1.2-10.8)
Controls without complications #, n=697	17 (2.4)	1.0 *
Preterm birth without other complications #, n=196	14 (7.1)	3.1 (1.5-6.4)

Abbreviations: CI, confidence interval.

* Reference group.

Women with pregnancies lasting ≥195 gestational days and without stillbirth, pre-eclampsia, IUGR, placental abruption, or chorionamnionitis (IUGR could be evaluated only for pregnancies lasting ≥195 gestational days).

8.5 FII G20210A in Studies I-IV

Table 12 summarizes genotype frequencies of FII G20210A in cases and controls in Studies I-IV and presents the overall risk associated with FII G20210A and the studied disease entity.

Table 12. *Genotype frequencies of FII G20210A in Studies I-IV.*

Genotype		Number of Cases (%)	Number of controls (%)	Odds ratio (95% CI)*
Venous thrombosis		**34**	**641**	
FII G20210A	GG	33	635	3.2 (0.07-27.6)
	GA	1 (2.9)	6 (0.9)	
	AA	0	0	
Pre-eclampsia		**248**	**679**	
FII G20210A	GG	244	673	1.8 (0.5-6.6)
	GA	4 (1.6)	6 (0.9)	
	AA	0	0	
Stillbirth		**44**	**776**	
FII G20210A	GG	44	769	-
	GA	0	7 (0.9)	
	AA	0	0	
Preterm birth		**324**	**752**	
FII G20210A	GG	323	745	0.3 (0.04-2.7)
	GA	1 (0.3)	7 (0.9)	
	AA	0	0	

Abbreviations: CI, confidence interval.

* Carriers of the FV Leiden (heterozygotes and homozygotes) versus wild-type.

Genotype: GG, wild-type homozygote; GA, F II G20210A heterozygote; AA, F II G20210A homozygote.

8.6 Other polymorphisms than FV Leiden and FII G20210A in Studies I-IV

The genotype frequencies of FV A4070G, PROC T8553G, MTHFR C677T, TFPI C536T, and FXIII V34L in Studies I-IV are presented in table 2 of each study (see original articles). In this study population, none of them was significantly associated with an increased risk for venous thrombosis, pre-eclampsia, stillbirth, or preterm birth. FXII V34L was not associated with a decreased risk for these disease entities.

8.7 Blood group in Studies I-IV

The prevalence of ABO and Rh blood groups in the Finnish population is as follows: O 31%, A 44%, B 17%, AB 8%, Rh D positive 87%, and Rh D negative 13%. Table 13 summarizes the distribution of ABO blood groups in cases and controls for each study.

Table 13. *The prevalence of ABO blood groups in cases and controls.*

	Number of Cases (%)	Number of controls (%)	Odds ratio (95% CI)
Venous thrombosis	**34**	**641**	
O	7 (20.6)	207 (32.3)	
A	18 (52.9)	278 (43.4)	
B	6 (17.6)	117 (18.3)	
AB	3 (8.8)	39 (6.1)	
Blood group O	7 (20.6)	207 (32.3)	1.0 *
Blood group Non-O	27 (79.4)	434 (67.7)	1.8 (0.8-4.3)
Pre-eclampsia	**248**	**679**	
O	72 (29.0)	217 (32.0)	
A	104 (41.9)	294 (43.3)	
B	40 (16.1)	124 (18.3)	
AB	32 (12.9)	44 (6.5)	
Blood group Non-AB	216 (87.1)	635 (93.5)	1.0 *
Blood group AB	32 (12.9)	44 (6.5)	2.1 (1.3-3.5)
Stillbirth	**44**	**776**	
O	20 (45.5)	243 (31.3)	
A	15 (34.1)	337 (43.4)	
B	7 (15.9)	140 (18.0)	
AB	2 (4.5)	56 (7.2)	
Blood group Non-O	24 (54.5)	533 (68.7)	1.0 *
Blood group O	20 (45.5)	243 (31.3)	1.8 (0.99-3.4)
Preterm birth	**324**	**752**	
O	97 (29.9)	239 (31.8)	
A	134 (41.4)	333 (44.3)	
B	65 (20.1)	131 (17.4)	
AB	28 (8.6)	49 (6.5)	
Blood group O	97 (29.9)	239 (31.8)	1.0 *
Blood group Non-O	227 (70.1)	513 (68.2)	1.1 (0.8-1.4)

Abbreviations: CI, confidence interval.

* Reference group.

9 DISCUSSION

Well-planned and conducted case-control studies can provide valuable information on the association between a risk factor and disease and they can be reliably used to test epidemiologic hypothesis [48]. A nested case-control study is a modification of a cohort study, in which only cases and a sample of controls in a fixed cohort are assessed in detail [8]. This nation-wide population-based nested case-control study assessing FV Leiden as a risk factor for selected pregnancy complications in Finland was conducted according to acknowledged principles of epidemiological studies. The adequate national registers provided exceptionally good preconditions.

9.1 Ethnic background

In genetic association studies, false positive and false negative associations may be possible due to population structure [50]. In this study, this was avoided – at least according to present knowledge. Although the Finnish population is not as genetically homogeneous as it was even lately thought to be [139,140], the cases and controls are of the same ethnic Caucasian origin and come equally from different parts of Finland. Genetic differences between European subgroups are smaller than between subpopulations of other ethnic origins [50]. Similarly to other populations of Caucasian origin, FV Leiden is prevalent in Finland, although not as frequent as in some European countries.

9.2 Prevalence of FV Leiden in Finland

The prevalence of FV Leiden was 2.2-2.6% in controls of Studies I-IV, and 2.4% in the population sample of 644 blood donors. These numbers are in agreement with other Finnish materials, where the prevalence of FV Leiden has been 2.1-2.9% in controls [141,142].

The reason for the *post hoc* finding of increased prevalence of FV Leiden in controls over the age of 35 is unclear. One could hypothesize that it may be due to improved embryo implantation [43] associated with FV Leiden in women whose fertility is otherwise decreasing. Improved embryo implantation is also supported by an observation of lower miscarriage-rate during the first trimester in FV Leiden carriers with an overall similar miscarriage-rate [44]. It is unlikely that FV Leiden would have affected the decision of controls to participate and the participation-rate was the same in every age-group.

9.3 Bias and confounding

Bias is defined as any systematic error that may cause an incorrect estimate of the association between exposure and outcome [143]. Selection bias was avoided by using the Hospital Discharge Register in the identification of cases and controls. Detection bias is unlikely, as FV Leiden has most certainly not influenced the reporting rate of complications to the Hospital Discharge Register. Information bias was minimized by collecting data from medical records and questionnaires

on standardized forms blinded for laboratory results. In general, cases with pregnancy complications may remember their pregnancy in more detail than controls with uneventful pregnancy [143]. However, in this study, recall bias is of less importance as outcomes were gathered from the Hospital Discharge Register and verified from medical records, and as exposure (FV Leiden) is inherited and was assessed by laboratory analysis. Missing cases may bias absolute risk estimates, but their impact on relative risks is minor.

Factors that are associated with both the exposure (FV Leiden) and outcome (studied disease entity) are confounding factors when the factor is not on the causal pathway [144]. In other words, confounding factor increases (or decreases) independently the risk for outcome, being at the same time associated with exposure. For example, previous venous thrombosis increases the risk for future venous thrombosis, but because FV Leiden may increase the risk for both of these thromboses, i.e., be causally associated, previous venous thrombosis is not a true confounding factor.

9.4 Strengths of the study

The strengths of the study are the population-based setting, large number of consecutive pregnancies within a short time-period (same diagnostic criteria and diagnostic tools), cases and controls from the same pregnant population of Caucasian origin, and strict diagnostic criteria. The laborious strategy of checking all medical records allowed exclusion of false positive findings in cases and false negative findings in controls. This also made subdivision of cases possible.

It is not likely that the pre-pregnancy knowledge of FV Leiden has influenced the results, because at the beginning of the study, only four hundred women of childbearing age were known to carry FV Leiden in the whole country. Presently, this kind of study population would hardly be obtainable.

The genotyping was performed at the Finnish Genome Center as a collaboration project. Genotyping methods for the seven polymorphisms were set up by Anna Rautanen, and were included in her thesis [137]. On average, the final success rate of genotyping these polymorphisms was very high, 99.5% [137]. In addition, all positive findings of FV Leiden and F II G20201A were confirmed with a method validated for diagnostic purposes in the Finnish Red Cross Blood Service. All results were concordant and all markers obeyed the Hardy-Weinberg equilibrium.

9.5 Weaknesses of the study

The study design did not allow assessment of fetal (paternal) genes in association with pregnancy complications. In case of prematurity, it seems that heritability of preterm birth is mostly transmitted through maternal genes and fetal (paternal) genotype is less important [145]. Due to the study design, the assessment of the risk for recurrent venous thrombosis, pre-eclampsia, stillbirth, or preterm birth was not possible. The family history of studied disease entities could not be reliably assessed.

Despite of the large cohort of pregnant women, the number of venous thrombosis cases and stillbirth cases was low. This is due to the rarity of these conditions and

all cases were invited to the study. Instead, pre-eclampsia and preterm birth are so common that only some of the cases were invited.

Participation rate is one of the problematic issues in epidemiologic studies. In this study, participation rate was relatively low among controls (44-51%). Considering the cases, participation rate was especially low among cases with stillbirth (54%), which may be due to strong emotions linked to the devastating occurrence. The cause of the low participation rate among cases with preterm birth (53%) can only be speculated. It may be due to severe health consequences stemming from prematurity in the infant, or, in the case of very late preterm birth, no consequences at all diminishing the motivation to participate. However, it is not likely that any factor connected to FV Leiden would have caused cases and controls to participate differently.

9.6 Missing and false positive diagnoses in the Hospital Discharge Register

It is possible that some of the studied diagnoses – especially preterm birth, but for some extent also pre-eclampsia – may have been omitted from the three diagnoses reported in the Hospital Discharge Register if the pregnancy had been associated with several complications. It seems more unlikely that venous thromboembolism or stillbirth would have been unreported. In addition, possible misclassification or miscoding of diagnoses cannot be excluded. In the case of pre-eclampsia and preterm birth, which are both common, the sample size of cases would nevertheless have been restricted. It is unlikely that the studied variables (e.g. FV Leiden) would have influenced possible misclassification or reporting rate of studied diagnoses to the register. The use of the National Medical Birth Register (maintained by the National Institute for Health and Welfare) in identification of preterm births and stillbirths could have minimized the risk for missing diagnoses.

The potentially dangerous bias caused by false positive diagnoses in cases was avoided by reviewing the medical records. Systematic scrutiny for false negative cases, i.e., women with studied clinical entities without proper ICD-code in the Hospital Discharge Register, would not have been feasible. This kind of misclassification would also have only a minor impact on the results.

The bias due to non-validated registry-based diagnoses is a known source of error in epidemiological studies [146]. Checking all medical records proved crucial, because 28% of the diagnoses of venous thrombosis in the Hospital Discharge Register were actually not associated with the index pregnancy, and 40% of pre-eclampsia did not fulfil the strict diagnostic criteria used in this study.

In this material, many false positive ICD-10 codes for venous thromboembolism were due to a past venous thrombosis instead of an acute thrombosis associated with the index pregnancy. The ICD-10 classification does not include a separate code for past thrombosis (status post thrombosis), which is often recorded as diagnosis for example when prophylactic anticoagulation is started. This appears to be a common source of false positive diagnoses also observed by others [147].

Very strict criteria for pre-eclampsia were used. Therefore, it is possible that some cases with mild true pre-eclampsia have been excluded due to a lack of

documentation in the medical records. Additionally, some mild pre-eclampsia cases may have been registered with other ICD-codes (e.g. pregnancy induced hypertension). In this study, missing cases are less important than false positive cases would have been. In case of severe pre-eclampsia, misreporting seems more unlikely. In a Danish study on the validity of registry diagnoses of pre-eclampsia, 95% of true pre-eclampsia cases were reported in the registry, but only about 70% were classified correctly as mild or severe pre-eclampsia [148].

9.7 Study I – Pregnancy-associated venous thrombosis

In this study, FV Leiden was associated with a 6 to 11-fold risk for pregnancy-associated venous thrombosis. Although FV Leiden was a major risk factor, the overall absolute risk of VTE for a FV Leiden carrier was estimated to be only 318 per 100,000. That is, one of 314 carriers would have thrombosis in association with pregnancy. On the population level, FV Leiden was estimated to be responsible for 19% of pregnancy-associated thromboses. The risk resulting from the combination of FV Leiden and older maternal age was additive, whereas the interaction of FV Leiden with BMI, and FV Leiden with non-O blood group resulted in a more than additive risk.

The risk associated with FV Leiden was of the same magnitude as in other studies (OR 3.7-18.3, see references in tables 1 and 2), although the studies vary in many respects. In a study by McColl *et al.* [59], the absolute risk of a carrier of FV Leiden for pregnancy-associated venous thrombosis was estimated to be slightly lower than in this study, 1 in 437. At the moment, except for the present study, no other studies estimating population attributable risk proportion of FV Leiden for pregnancy-associated venous thrombosis exist. In the general population, a study on White men and women aged 18-65 years estimated that 6.6% of VTE episodes were attributable to FV Leiden [149].

Pregnancy-associated venous thromboembolism is rare, occurring usually in less than 1 in 1,000 pregnancies in western countries [58-62]. In this study, objectively verified VTE was found in 34 per 100,000 pregnancies. If thromboses in the index pregnancies occurred at the same frequency in participants and non-participants, due to 80% participation rate, this study missed nine true pregnancy-associated venous thromboses. Taking this into account, the incidence of 43 per 100,000 is still less than in other studies where the incidence of objectively verified venous thromboembolism has ranged from 61 per 100,000 deliveries in mainly Hispanic population [62] to 85 per 100,000 in Danish population [60], and to 86-123 per 100,000 in UK [40,59]. In studies with register-based diagnoses, the incidence has been 85 per 100,000 deliveries in the UK (23% non-European origin) [60,61], and in the USA, 107 per 100,000 in Asian women, 125 per 100,000 in Hispanic women, 175 per 100,000 in White women, and 264 per 100,000 in Black women [150]. The detected incidence of pregnancy-associated VTE varies in different studies probably due to differences in the diagnostic tools, due to whether the incidence is based on register-based data or data from medical records, due to whether the incidence is calculated for pregnancies (as in this study) or for deliveries (as in most of the other studies) and due to true differences in the incidence of VTE in different populations. It seems unlikely that deep venous thromboses, pulmonary emboli, and cerebral venous thromboses would have been substantially underreported in the Hospital Discharge Register.

In the 70 reviewed medical records, diagnosis of any venous thrombosis was recorded in association with the index pregnancy. However, in 20 women, the venous thrombosis had occurred clearly before the index pregnancy. These women along with women with non-objectively diagnosed thrombosis (4), and women with superficial venous thromboses (12) were excluded from this study. Therefore, without proper assessment of the diagnoses by reviewing the medical records, the results could have been strongly misleading. In a Danish study evaluating discharge diagnoses of VTE during pregnancy and puerperium, the positive predictive value was 79% when focusing on confirmed VTE in association with pregnancy [146]. The authors concluded that diagnoses are recommended to be verified by a review of the medical records in epidemiological studies assessing pregnancy-related venous thrombosis.

Even if FV Leiden can be regarded as an established risk factor for venous thrombosis, there still is need for well-designed large prospective population-based studies to further clarify the absolute risk of pregnancy-related venous thrombosis in non-selected carriers of FV Leiden.

9.8 Study II – Pre-eclampsia

In this study, FV Leiden was associated with a 1.7-fold risk for pre-eclampsia, but the association was not statistically significant. The study would have had sufficient power (80% power, 5% α-error) to detect a 3-fold risk associated with FV Leiden.

The point estimate of the risk in this study is in the range of other studies. In three meta-analyses of mainly case-control studies [79,92,93], FV Leiden has been associated with about a 2-fold risk whether all or only severe pre-eclampsia has been considered, or whether heterozygous and homozygous carriers of FV Leiden have been analyzed combined or separately. In one meta-analysis of six cohort studies, FV Leiden was associated with a 1.5-fold risk for pre-eclampsia [96]. On the contrary, the meta-analysis of nine prospective cohort studies by Rodger *et al.* did not show significant association between FV Leiden and pre-eclampsia, the pooled OR being 1.2 [3], as did not the most recent nested case-control study by Kahn *et al.* [97].

In the whole study population, 1,084 of 100,000 (1.1%) women had the diagnosis of pre-eclampsia, eclampsia, or both in the Hospital Discharge Register. This incidence is somewhat lower than the incidence of 1.5-2.9% in recent European studies where diagnoses were verified from the medical records [40,148,151,152]. This may be due to the fact that only three diagnoses per hospital care period are recorded in the Hospital Discharge Register. For comparison, in the Medical Birth Register, which includes ten diagnoses from the period of pregnancy and ten diagnoses from the period of delivery, the incidence of pre-eclampsia (including eclampsia) has been 2.0% in 2006 and 2007 (personal communication, Eija Vuori, October 2008). It is possible that pre-eclampsia may have been omitted from the three diagnoses in the Hospital Discharge Register if the pregnancy had also been associated with several other complications and if the pre-eclampsia had been mild. Mild pre-eclampsia may also have been miscoded. The difference in incidence rates may also be explained by different definitions of pre-eclampsia, different methods of collecting data, whether only primigravid women were included, and whether the incidence was proportioned for pregnancies or for deliveries.

From the clinical point of view, mild pre-eclampsia is usually more common than severe pre-eclampsia. In this study, however, 68% of the cases had severe pre-eclampsia. This may be due to the reasons already described and due to the very strict criteria for pre-eclampsia used in this study, which may have led to the exclusion of some true mild cases of pre-eclampsia if the documentation in the medical records were insufficient. The finding is not unique, as in a recent study by Klemmensen *et al.* [148], 63% of strictly defined pre-eclampsia cases were severe. The incidence of severe pre-eclampsia is in agreement with present knowledge, which supports the idea of mild pre-eclampsia being underreported. In this study, the estimated incidence of severe pre-eclampsia in the whole study population was 442 per 100,000 pregnancies, which is in line with incidences of 516 per 100,000 [153] and 458 per 100,000 (mainly White) [154] reported in the UK.

Despite the limitations of meta-analyses and individual studies, FV Leiden seems not to be a major risk factor for pre-eclampsia. However, as pre-eclampsia is a very heterogeneous disease entity, it may be worth further studying well-specified clinical subgroups separately in ethnically homogeneous populations to minimize the risk of not observing any true association with FV Leiden.

9.9 Study III – Stillbirth

FV Leiden was associated with a 3-fold risk for unexplained stillbirth in all and in singleton pregnancies, and a 4-fold risk for late unexplained stillbirth. About one in ten of the mothers with unexplained stillbirth was a carrier of FV Leiden.

Three meta-analyses [79,92,106] have shown similar, a 2- to 3-fold risk for association between FV Leiden and late fetal loss. However, studies vary considerably regarding the selection of cases and controls, definition of stillbirth, and what other possible causes of stillbirth have been excluded. Published case-control studies of unexplained stillbirth (table 3) have mainly found a statistically significant association with FV Leiden, but retrospective cohort studies (table 4) of all stillbirths mostly have not. In many cohort studies [86,115,116,118], the stillbirth rate in FV Leiden non-carriers has been unexpectedly high, 2-5% (6-11% in FV Leiden carriers) compared with the estimated rate of 0.4-0.7% in developed countries [98]. Two of these studies [115,116] assessed cohorts from thrombophilic families. The only prospective antenatal cohort study [105], despite its limitations of heterogenic ethnicity and small number of unexplained stillbirths, had a reliable stillbirth rate (0.35%) and found nearly a 9-fold risk associated with FV Leiden.

In the whole study population, 224 of 100,000 women had the diagnosis of stillbirth in the Hospital Discharge Register. This incidence of 2.2 per 1,000 pregnancies is somewhat lower than the estimated incidence of 4.2-6.8 per 1,000 deliveries in developed countries [98], but it is in agreement with the low stillbirth rate in the Nordic countries [99]. The review of medical records of the 120 participants revealed that in 20% of cases fetal death had occurred before 22nd gestational week, or the diagnosis was miscoded. The participation rate was relatively low, which may be due to strong emotions associated with the stillbirth. However, it is not likely that FV Leiden or some factor associated with it would have affected the decision of participation.

Since association of FV Leiden with stillbirth varies in different studies, it is likely that other factors modulate the risk for placenta-mediated pregnancy complications, such as fetal loss and pre-eclampsia. Recent data from mouse models have shown that, at least in mice, fetal thrombophilia expressed on trophoblast cells increases the risk for fetal loss in carriers of FV Leiden [155]. Also, the mechanism by which thrombophilia increases the risk for fetal loss is not always via placental thrombosis, but through the impairment of placental growth and morphogenesis [155].

The "ultimate truth" about the strength of association between FV Leiden and unexplained stillbirth remains to be seen with results from future prospective cohort studies. Future studies should preferably analyze both maternal and fetal (paternal) genotypes to assess factors that modify the risk associated with FV Leiden.

9.10 Study IV – Preterm birth

In this study, FV Leiden was associated with a 2.5-fold risk for preterm birth in all as well as in singleton pregnancies. FV Leiden was especially associated with late preterm birth (a 3-fold risk) but not with early preterm birth. FV Leiden was consistently associated with an increased risk also in subgroup analyses of women with a singleton pregnancy, women without other pregnancy complications, and women with spontaneous preterm delivery.

At present, this study is the largest population-based study on the association between FV Leiden and preterm birth. Other studies assessing the association are few and mostly small (tables 5 and 6). Differences in study populations, ethnic backgrounds, and exclusion criteria make comparison with the present study difficult.

The three prior studies with positive findings may be considered preliminary. The first positive association came from a study of very low birth weight infants [125], but could not be confirmed later by the same group [130]. The second positive observation came from 50 women with preterm birth among whom the prevalence of FV Leiden was higher than in general population [126]. The third positive result came from the only prospective cohort study existing today, albeit from a subanalysis of women with preterm birth and evidence of placental hemorrhage [127].

Also the four studies with negative findings have limitations that restrict the generalizability of the results. First, the study population being other than Caucasian [128], or ethnic background not being reported [129,131,132]. Second, studying only a subgroup of preterm birth: women with VLBW preterm infants [130], or women with preterm infants admitted to neonatal intensive care unit and women with term infants admitted to neonatal intensive care unit used as controls [129]. Third, being very small [131]. In an otherwise representative study [112], the prevalence of FV Leiden was very variable in matched controls of different disease entities (2 to 8.2%) leading to a very high risk estimate for stillbirth but resulting in no association with preterm birth.

The incidence of preterm birth is about 5% in Finland [99]. However, in the whole study population, only 1,402 of 100,000 (1.4%) women had the diagnosis of preterm birth in the Hospital Discharge Register. As in the case of pre-eclampsia, this may be due to the fact that only three diagnoses per a hospital care period are recorded in the Hospital Discharge Register. It is possible that preterm birth may have been omitted from the three diagnoses in the Hospital Discharge Register if the pregnancy had also been associated with several other complications, or if the preterm birth had occurred near term. The participation rate of preterm birth cases was relatively low. Speculatively, this may be due to devastating consequences of prematurity, or no consequences at all diminishing the motivation to participate. It is, however, very unlikely that FV Leiden would have affected the reporting rate to the register or the decision of cases to participate.

Preterm birth is a very heterogeneous entity, and the pathophysiology behind it may differ in early and late preterm birth [119]. In this study, FV Leiden was associated with late but not with early preterm birth. Early preterm birth was associated significantly more often with chorionamnionitis, premature rupture of membranes, and IUGR (data not shown). Uterine anomaly was also more common in early preterm birth, although not statistically significantly. The prevalence of pre-eclampsia, in vitro fertilization, and twin pregnancy was equal in early and late preterm birth. As inflammation is involved in both term and preterm delivery [5], and as coagulation and inflammation are interrelated [6], it is tempting to speculate that FV Leiden (thrombophilia) through excess thrombin production would lead to preterm birth by prematurely activating inflammatory mechanisms that normally induce term labour.

As no unselected prospective studies on the association of FV Leiden with preterm birth exist, further well-designed, preferably prospective studies are needed to establish the association in Caucasian population.

9.11 Does FV Leiden have causal influence on pregnancy complications?

Hill's criteria from 1965 have been widely used to evaluate the causality of a risk factor for a disease, although it has been said that in his own opinion none of these viewpoints can absolutely prove or disprove a causal effect [156]. Hill's criteria are: 1) strength of association, 2) consistency of observed association, 3) temporality, 4) specificity, 5) biologic plausibility, 6) biologic gradient, 7) coherence, 8) analogy, and 9) experiment.

9.11.1 FV Leiden as risk factor for pregnancy-associated venous thrombosis

1) In this study, FV Leiden was associated with an 11-fold increased risk for pregnancy-associated thrombosis and a 6-fold risk for first venous thrombosis. Association can be considered strong.

2) Association of FV Leiden with pregnancy-associated venous thrombosis has been consistent. In other studies FV Leiden has been associated with a 4.5-fold to 18-fold risk. In a pooled analysis, the risk was 8-fold.

3) As FV Leiden is inherited, temporal relationship exists.

4) All carriers of FV Leiden do not get venous thrombosis in association with pregnancy or even in their life-time. On the other hand, FV Leiden may also be associated with other disease entities. Venous thrombosis has many other risk factors as well and FV Leiden is not necessary for venous thrombosis to occur.

5) Biologic plausibility exists as FV Leiden changes the structure of factor V making it resistant to protein C and unable to function as a cofactor for protein C. These functional changes lead to increased formation of thrombin.

6) Homozygotes were too rare to assess biologic gradient. However, dose-effect has been demonstrated in other studies, in which the risk for thrombosis has been much higher in homozygotes than in heterozygotes (34-fold vs. 8-fold).

7) FV Leiden as a risk factor for pregnancy-associated venous thrombosis does not contradict the present knowledge of FV Leiden increasing the risk for venous thrombosis in a biologically plausible way.

8) In addition, other thrombophilias have been found to increase the risk for pregnancy-associated venous thrombosis.

9) Anticoagulant prophylaxis during pregnancy and puerperium is proven to protect against pregnancy-associated thrombosis.

The point 4 is not entirely fulfilled, but there is no doubt that FV Leiden causally increases the risk for venous thrombosis. However, FV Leiden is only one of the risk factors for the multifactorial disease of venous thrombosis. Thrombosis occurs when the effect of simultaneous risk factors reaches a certain trigger point.

9.11.2 FV Leiden as risk factor for pre-eclampsia, stillbirth, and preterm birth

1) In this study, FV Leiden was associated with trend of a 1.7-fold risk for pre-eclampsia, 3-fold risk for unexplained stillbirth, and 2.5-fold risk for preterm birth. Association can be considered weak for pre-eclampsia and modest for stillbirth and preterm birth.

2) Association of FV Leiden with these placenta-mediated pregnancy complications has been inconsistent, possibly due to the heterogeneity of the studies.

3) As FV Leiden is inherited, temporal relationship exists.

4) All carriers of FV Leiden do not get placenta-mediated complications. On the other hand, FV Leiden is also associated for example with venous thrombosis. Placenta-mediated pregnancy complications have heterogeneous origin with unknown pathophysiological mechanisms, and FV Leiden is not necessary for them to occur.

5) Biologic plausibility exists as FV Leiden changes the structure of factor V making it resistant to protein C and unable to function as a cofactor for protein C. These functional changes lead to an increased formation of thrombin. Coagulation and inflammatory mechanisms are closely interrelated and it is possible that increased thrombin formation could interfere with the balance of inflammatory mechanisms during pregnancy.

6) Rarity of homozygotes hampers the assessment of biologic gradient.

7) FV Leiden as a risk factor for placenta-mediated pregnancy complications does not contradict the present knowledge of FV Leiden increasing the risk for venous thrombosis in a biologically plausible way.

8) Other thrombophilias (especially antiphospholipid antibodies) have also been suggested to increase the risk for placenta-related pregnancy complications.

9) Anticoagulant prophylaxis during pregnancy and puerperium has not been proven to prevent placenta-mediated pregnancy complications. Trials are ongoing.

As concluded by Rodger *et al.* [157], causality between FV Leiden and placenta-mediated pregnancy complications has not been proven. However, with the present knowledge, the modifying effect of FV Leiden cannot be ruled out. A mouse model has given novel experimental support to the idea of causality [158]. In that experiment, fetal gene defects expressed in trophoblast cells of the placenta modified the risk of fetal loss in FV Leiden carriers [158].

It can be noted that the risk associated with FV Leiden is consistently much higher for venous thrombosis than for specific pregnancy complications. This difference in the strength of association may be a sign of different pathophysiological mechanisms behind these disease entities.

10 CONCLUSIONS AND FUTURE PERSPECTIVES

This large population-based nested case-control study showed FV Leiden to be a clear risk factor for many pregnancy complications. The study was possible due to the adequate national registers, due to legislation allowing the use of registers in research, and due to positive attitude of the Finnish women towards research. Checking all medical records proved crucial for reliable results. The codes for venous thrombosis and pre-eclampsia in the Hospital Discharge Register, in particular, included false positive diagnoses considering the diagnostic criteria used in this study.

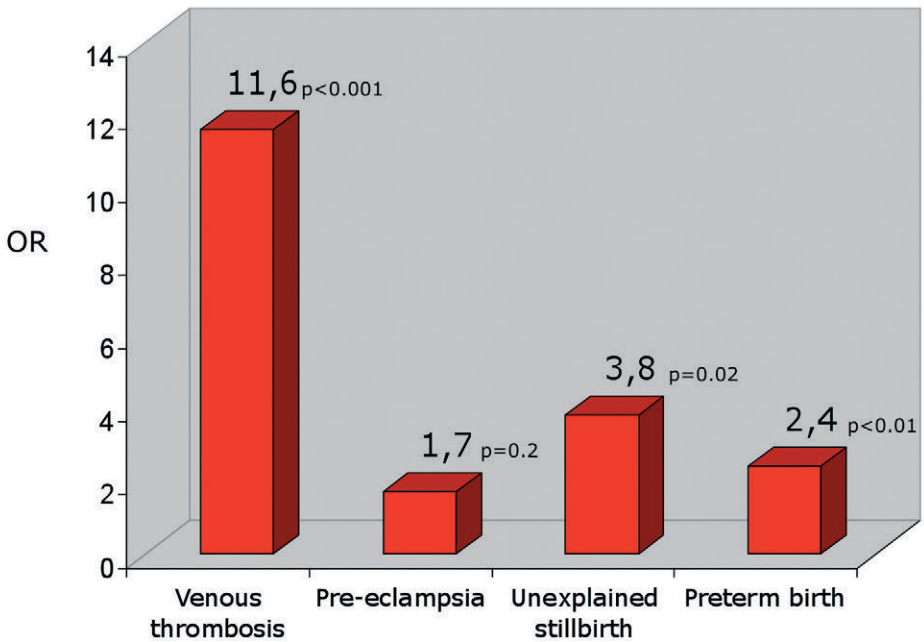
As expected, FV Leiden was a strong risk factor for pregnancy-associated venous thrombosis. Maternal carriage of FV Leiden was associated with an 11-fold risk. When analyzing only cases with the first venous thrombosis, FV Leiden was associated with a 6-fold risk. The risk was multiplied when the carriage of FV Leiden was associated with non-O blood group, BMI over 30 kg/m^2, or age over 35 years. These results suggest interaction between FV Leiden and common risk factors for venous thrombosis. In the whole study population, 19% of thromboses were attributable to FV Leiden. The results confirmed and extended prior results of the association between FV Leiden and pregnancy-associated venous thrombosis.

When evaluating pre-eclampsia, FV Leiden was associated with a 1.7-fold risk. The point estimates of the risk in subgroups of pre-eclampsia were 1.5-2.5 when all women were analyzed and 2.4-3.4 when primigravid women were considered. However, these associations were not statistically significant. In conclusion, the results suggest that the association between FV Leiden and pre-eclampsia, if any, is weak.

Novel information was gained especially on unexplained stillbirth and preterm birth. When evaluating unexplained stillbirth, FV Leiden was associated with over a 3-fold risk. FV Leiden was especially associated with late unexplained stillbirth with about a 4-fold risk in both all and singleton pregnancies. When evaluating preterm birth, FV Leiden was associated with over a 2-fold risk. FV Leiden was especially associated with late preterm birth with about a 3-fold risk. When primigravid cases and controls were analyzed, the risk was about 3-fold. When analysis was restricted to cases and controls without stillbirth, pre-eclampsia, IUGR, placental abruption, or chorionamnionitis, the risk associated with FV Leiden was still over 2-fold. In conclusion, the results show that FV Leiden is associated with an increased risk for both unexplained stillbirth and preterm birth but is not the major risk factor for these disease entities.

The results are in accordance with the current guidelines for screening and prophylaxis. Future studies are needed to show if the association between FV Leiden and specific pregnancy complications is causal and if prophylaxis with LMWH has a beneficial effect. Until then, screening for FV Leiden and prophylactic treatment of women with pregnancy complications like pre-eclampsia, stillbirth, or preterm birth is not recommended. However, screening for FV Leiden in women with personal or family history of venous thrombosis may be beneficial regarding thrombosis prophylaxis in association with future pregnancies.

Figure 4. *Association of FV Leiden with pregnancy complications.*



In conclusion, maternal carriage of FV Leiden was associated with a strong risk for pregnancy-associated deep venous thrombosis, a trend of increased risk for pre-eclampsia, and a moderate risk for unexplained stillbirth and preterm birth. The results can be generalized to Finnish women with pregnancies continuing beyond the first trimester and may be applied to Caucasian women in populations with a similar prevalence of FV Leiden and high standard prenatal care.

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Leena Hiltunen

12 APPENDIX

ICD-10 codes from the Hospital Discharge Register

G08	Phlebitis/endophlebitis/thrombophlebitis septica sive thorombosis/embolia septica venarum intracranialium/intraspinalium
G45	Ischaemia cerebri transitoria
G45.0-G45.9	
G45.3	Amaurosis fugax
G95.1	Myelopathiae vasculares (infarctus medullae spinalis acutus, thrombosis arterialis medullae spinalis, thrombosis/thrombophlebitis intraspinalis non pyogenes)
H34	Occlusiones vasculares retinae
H34.0-H34.9	
I20	Angina pectoris
I20.0-I20.99	
I21	Infarctus myocardii acutus
I21.0-I21.99	
I24.0	Occlusio/embolia/thromboembolia arteriae/venae coronariae sine infarctu myocardii
I26	Embolia pulmonalis
I26.0-I26.9	
I51.3	Thrombosis intracardialis non alibi classificata
I63	Infarctus cerebri
I63.0-I63.9	
I63.6	Infarctus (non pyogenes) cerebri e thrombosi venorum cerebralium
I65	Occlusio sive stenosis arteriarum praecerebralium sine infarctu cerebri
I65.0-I65.9	
I66	Occlusio sive stenosis arteriarum cerebralium sine infarctu
I66.0-I66.9	
I67.6	Thrombosis non pyogenes systematis venosi intracranialis
I74	Embolia/thrombosis arterialis
I74.0-I74.9	
I80	Phlebitis et thrombophlebitis
I80.0-I80.9	Phlebitis/thrombophlebitis venarum superficialium membrorum inferiorum/ venae femoralis/ venae iliacae/ venarum profundarum membrorum inferiorum/ membri inferioris non specificata/ aliis locis specificatis/ loco non specificato)
I81	Thrombosis venae portae

I82	Embolia/thrombosis aliarum venarum
I82.0-I82.9	Syndroma Budd-Chiari, thrombophlebitis migrans, embolia/thrombosis venae cavae superioris/ venae cavae inferioris/ venae cavae/ venae renalis/ venae subclaviae/ aliarum venarum specificatarum/ venae non specificatae)
K55.0	Morbositates vasculares intestini acutae (embolia/thrombosis arteriae/venae mesentericae superioris/inferioris)
N28.0	Ischaemia renis (embolia/thrombosis arteriae renalis)
O03	Abortus spontaneus
O03.0-O03.9	
O06	Abortus non specificatus
O06.0-O06.9	
O08.2	Embolia post abortum sive graviditatem extrauterinam sive molam hydatidosam (Embolia pulmonalis)
O08.7	Aliae complicationes venosae post abortum sive graviditatem extrauterinam sive molam hydatidosam
O14	Hypertensio gestationalis cum proteinuria significanti
O14.0	Prae-eclampsia moderata
O14.1	Prae-eclampsia gravis
O14.9	Prae-eclampsia non specificata
O15	Eclampsia
O15.0	Eclampsia in graviditate
O15.1	Eclampsia inter labores
O15.2	Eclampsia in puerperio
O15.9	Eclampsia tempore non specificata
O22	Complicationes venosae in graviditate
O22.2	Thrombophlebitis superficialis in graviditate
O22.3	Thrombophlebitis profunda in graviditate
O22.5	Thrombosis venae cerebralis in graviditate
O22.8	Aliae complicationes venosae specificatae in graviditate
O22.9	Phlebitis in graviditate NAS
O36.4	Cura matris propter mortem intrauterinam fetus
O36.5	Cura matris propter retardationem crescendi fetus
O60	Partus praematurus
O87	Complicationes venosae in puerperio
O87.0	Thrombophlebitis superficialis in puerperio
O87.1	Thrombophlebitis profunda in puerperio
O87.3	Thrombosis venae cerebralis in puerperio

O87.9	Complicatio venosa non specificata in puerperio (Phlebitis/thrombosis puerperalis NAS)
O88	Embolia obstetrica
O88.2	Embolia thrombotica obstetrica (Embolia pulmonalis obstetrica/puerperalis NAS)
O95	Mors obstetrica matris causa non specificata
O96	Mors ex qualibet causa obstetrica occurrens plus quam XLII dies sed minus quam unum annum post partum
O99.4	Morbi systematis circulatorii complicantes graviditatem, partum et puerperium
O99.5	Morbi systematis respiratorii complicantes graviditatem, partum et puerperium
Z37.1	Partus fetus mortuus
Z37.3	Partus fetus mortuus
Z37.4	Partus fetus mortuus
Z37.6	Partus fetus mortuus
Z37.7	Partus fetus mortuus

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